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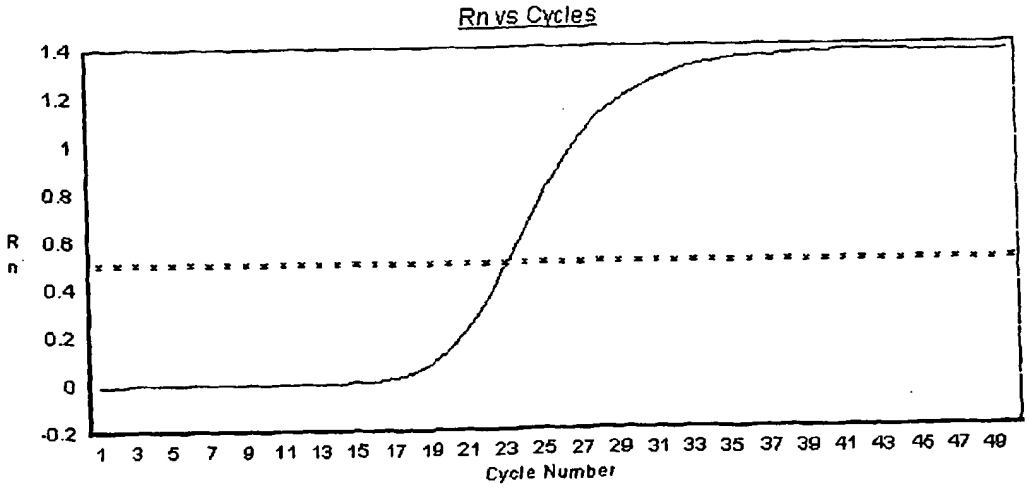
(54) Title: ASSAY



(57) Abstract: The invention provides a polynucleotide having the sequence of SEQ ID NO:1 (CCTCGGGGTACCTGAAGGGCATCC) or a fragment thereof, which fragment is capable of binding specifically to the complementary sequence of SEQ ID NO: 1, a polynucleotide having the sequence of SEQ ID NO:2 (CAC{T/C}T{T/C}AAG{G/A}TGACA{T/C}TG{G/A}TACTG GTAC) and a polynucleotide having the sequence of SEQ ID NO:3 (CAGAT{C/T}CC{G/A}AGTG{T/A}C{I}C{I}TGTTA). The invention also provides a method for determining whether a sample contains FMDV comprising (a) contacting the sample with a probe comprising a polynucleotide having the sequence of SEQ ID NO:1 (CCTCGGGGTACCTGAAGGGCATCC) or a fragment thereof, which fragment is capable of binding specifically to the complementary sequence of SEQ ID NO: 1, *in vitro* under conditions that allow the probe to bind specifically to a target polynucleotide; and (b) determining whether the probe has bound to a target polynucleotide; thereby to determine whether the sample comprises a target polynucleotide, the presence of a target polynucleotide in a sample being indicative of presence of FMDV.

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ASSAY

The present invention relates to means and methods for detection of Foot-and-Mouth Disease Virus.

Foot-and-Mouth Disease (FMD) is a highly contagious viral infection of domestic and wild cloven-hoofed animals, such as pigs and wild and domesticated ruminants such as cattle, buffalo, sheep, goats and deer.

FMD virus (FMDV) is a member of the *Aphthovirus* genus in the *Picornavirdae* family of positive sense, single-stranded RNA viruses (Belsham, *Prog Biophys Mol Biol*, 1993, **60**(3): 241-260). There are seven serotypes (A, O, C, Asia1, SAT1, SAT2 and SAT3) and over 65 subtypes are known (Martinez et al, 1991, *Virology*, **184**: 695-706). The virus has a high mutation rate (Haydon et al, 1998, *Virus Genes*, **16**(3): 253-266), changing, on a random basis, between one and eight nucleotides per replication (Knowles *et al*, 3 March 2001, *The Veterinary Record*: 258-259).

FMD can spread rapidly and result in serious economic consequences. It can cause high mortality in young animals and production losses in adults, and is considered the single most important constraint to trade in live animals and animal products. Spread of FMD can occur by a variety of mechanisms including animal movement, contaminated animal products (meat, milk, semen), mechanically by people and fomites, and by the wind (Knowles *et al*, 3 March 2001, *The Veterinary Record*: 258-259).

Whilst the high genetic variability of FMDV can be useful in characterising and tracking individual strains of FMDV, it also present problems. For example, it is not possible to use a single vaccine to immunise animals

against all FMDV strains. This was demonstrated by Dawe *et al* (1994, *The Veterinary Record*, **134**: 211-215) where it was shown that, despite immunising cattle against the SAT2 serotype of FMDV, the cattle were still susceptible to SAT2 after it had been propagated in buffalo for five months. Dawe *et al* (1994, *The Veterinary Record*, **134**: 230-232) also demonstrated that, despite being immunised against SAT1, cattle were not immune to SAT1 from wild buffalo. This demonstrates the high level of variability exhibited by FMDV, even between isolates of the same serotype.

Fast and reliable diagnosis of FMD is essential for effective disease control. Rapid and sensitive FMDV diagnosis is required to enable the appropriate control measures to be implemented to restrict spread of infection and eradicate disease (Reid *et al*, 1998, *Journal of Virological Methods*, **70**: 213-217).

Conventionally, primary virus diagnosis of FMD is carried out by Enzyme-Linked Immunosorbent Assay (ELISA) (Ferris and Dawson, 1988, *Vet Microbiol*, **16**: 201-209) on suspensions of clinical samples. In some cases it may be necessary to multiply the virus by infecting cells in culture, in which case it can take up to four days to be sure that no virus is present (Knowles *et al*, 3 March 2001, *The Veterinary Record*: 258-259).

Attempts to speed up detection have been unsuccessful. Rapid techniques such as reverse transcription polymerase chain reaction (RT-PCR) have been employed in the detection of FMD virus genomic RNA in samples. RT-PCR requires the use of primer pairs that will bind to, and amplify, complementary target polynucleotides. The variability of FMDV has prevented a single effective RT-PCR assay being developed for all FMDV serotypes and subtypes. Serotype-specific primer pairs have been produced

(Reid *et al*, 1999, *Journal of Virological Methods*, **83**: 113-123), but each pair was able to detect only the serotype it was specific for.

Attempts to develop 'universal' primers have been unsuccessful. The use of 'universal' primers by Reid *et al* (1998, *Journal of Virological Methods*, **70**: 213-217) resulted in failure to identify infection in 13 out of 80 positive samples and the identification of 11 false positives out of 86 negative samples.

More recently, Reid *et al* (2000, *Journal of Virological Methods*, **89**: 167-176) reported that, whilst primers had been developed that could successfully detect O, A, C and Asia 1 serotypes, detection of SAT1, SAT2, and SAT3 serotypes was less efficient. Moreover, the sensitivity of the RT-PCR method was 100-fold lower than conventional diagnostic methods. This led the authors to conclude that the benefits of reliability, robustness and ease of test performance will ensure the retention of existing ELISA-based procedures, and that RT-PCR could be used merely as a support to the conventional methods.

To date, the only successfully used RT-PCR method suggested for diagnosing FMD has additionally involved an ELISA step (Alexandersen *et al*, *Journal of Clinical Microbiology*, 2000, **38**(12): 4604-4613). This technique necessitates the denaturation of the PCR product, hybridisation with a labelled probe, fixation to form complex onto a solid phase, multiple washes, incubation with a labelled antibody conjugate and yet further washes before measurement of the amount of antibody conjugate binding in order to diagnose the presence or absence of FMDV in a sample. Thus the technique is slow and comprises a many steps at which inconsistencies can occur. Furthermore, the technique described in Alexandersen *et al*, *Journal of Clinical Microbiology*, 2000, **38**(12): 4604-4613 did not produce a probe

that was homologous for all FMDV strains. Instead the technique used 'forcing' conditions in order to take advantage of the fact that, under very high target and probe concentrations, binding will occur even with a number of mismatches between probe and target. Thus, under the conditions used, the probe could also bind to other highly homologous contaminating polynucleotides and result in false positives.

It is thus an object of the present invention to provide the means and methods for rapidly diagnosing FMDV. It is also an object of the present invention to provide an assay that is capable of detecting all FMDV serotypes and substantially all isolates thereof. It is also an object of the present invention to provide an assay with high sensitivity. It is also an object of the present invention to provide an assay that is reliable. It is also an object of the present invention to provide an assay that is specific. It is also an object of the present invention to provide an assay that is consistent.

According to the first aspect of the invention, there is provided an isolated polynucleotide having the sequence of SEQ ID NO: 1 (as shown below) or a fragment thereof, which fragment is capable of binding specifically to the complementary sequence of SEQ ID NO: 1. We have found that the 24 nucleotide sequence of SEQ ID NO: 1 is conserved in the genome of all examined FMDV serotypes and subtypes thereof, and can be used as a probe in diagnostic tests for FMDV.

SEQ ID NO:1

CCTCGGGGTACCTGAAGGGCATCC

By 'isolated' we mean that the polynucleotide is not located in a cell, *i.e. in situ*, but is suitable for *in vitro* use in the methods of the invention (see below).

Whilst the skilled person will appreciate that the polynucleotide according to the first aspect of the invention can be used in any suitable diagnostic method known in the art, the preferred method is a TaqMan™ RT-PCR assay (PE Applied Biosystems, Warrington, Cheshire, UK). Preferably the polynucleotide of the first aspect of the invention is used as a TaqMan™ probe.

The TaqMan™ method exploits the 5'-3' exonuclease activity of Taq DNA Polymerase to allow direct detection of the generation of PCR product.

In PCR, forward and reverse primers hybridise to specific sequences of target polynucleotide, resulting in it the amplification of those specific sequences and the intervening region. The TaqMan™ probe hybridises to a target sequence within the PCR product. When the PCR product is amplified in a subsequent cycle of PCR, Taq DNA Polymerase degrades the TaqMan™ probe (5'-3' exonuclease activity) so that the enzyme can continue to copy its target sequence. The degradation of the probe can be monitored and used as an indication of the generation of PCR product.

SEQ ID NO: 1 has a relatively high level of homology to genes in public databases, especially sequences involved in RNA interactions, such as promoter regions, RNA polymerase and 16S Ribosomal RNA. However, the maximum homology between the 24 nucleotide sequence of SEQ ID NO:1 and these other sequences is 18 nucleotides in a row, or 19 out of 20 in a single case. This is shown in the table below.

Sequence	Score	Length	Expect	Accession Number
Synechococcus sp. PS718 RNA polymerase subunit (rpoC1) gene, partial cds	36	18	0.30	AF245151
Synechococcus sp. PS677 RNA polymerase subunit (rpoC1) gene, partial cds	36	18	0.30	AF245136
Synechococcus sp. PS672 RNA polymerase subunit (rpoC1) gene, partial cds	36	18	0.30	AF245129
Synechococcus sp. CC9703 RNA polymerase subunit (rpoC1) gene, partial cds	36	18	0.30	AF153338
Homo sapiens clone RP11-296A19, complete sequence	32	16	4.7	AC012591
Porphyromonas gingivalis ATCC 33277 16S ribosomal RNA gene, complete	32	16	4.7	L16492
Archaeoglobus fulgidus, complete genome	32	16	4.7	NC_000917
Porphyromonas gulae strain B 243 16S ribosomal RNA gene, partial sequence	32	16	4.7	AF285874
Porphyromonas gulae strain chien 4.2 16S ribosomal RNA gene, partial seq	32	16	4.7	AF285873
Porphyromonas gulae strain ours 3.1 16S ribosomal RNA gene, partial sequence	32	16	4.7	AF285872
Porphyromonas gulae strain chat 3.1 16S ribosomal RNA gene, partial sequence	32	16	4.7	AF285871
Porphyromonas gingivalis 16S ribosomal RNA gene, partial sequence	32	16	4.7	AF285870
Porphyromonas gulae strain Manga 1.41 16S ribosomal RNA gene, partial seq	32	16	4.7	AF285869
Porphyromonas gingivalis 16S ribosomal RNA gene, partial sequence	32	16	4.7	AF287987
Porphyromonas gulae 16S ribosomal RNA gene,	32	16	4.7	AF287986

Sequence	Score	Length	Expect	Accession Number
partial sequence				
Synechococcus sp. PS729 RNA polymerase subunit (rpoC1) gene, partial cds	32	16	4.7	AF245147
Synechococcus sp. PS727 RNA polymerase subunit (rpoC1) gene, partial cds	32	16	4.7	AF245143
Porphyromonas gulae 16S ribosomal RNA gene, partial sequence	32	16	4.7	AF208290
Uncultured bacterium BURTON-18 16S ribosomal RNA gene, partial sequence	32	16	4.7	AF142838
Homo sapiens chromosome 16, BAC clone 472H7 , complete sequence	32	16	4.7	AC006112
Mus musculus potassium channel isoforms Merg1a and Merg1a' (Merg1) gene	32	16	4.7	AF012870
Synechococcus sp. CC9305-3 RNA polymerase subunit (rpoC1) gene, part cds	32	16	4.7	AF013610
Synechococcus sp. CC9317 RNA polymerase subunit (rpoC1) gene, partial cds	32	16	4.7	AF013609
Archaeoglobus fulgidus section 139 of 172 of the complete genome	32	16	4.7	AE000968
Gloeobacter violaceus DNA-dependent RNA polymerase (rpoC1) gene, part cds	32	16	4.7	U52340
P.gingivalis gene for 16S rRNA	32	16	4.7	X73964
Porphyromonas gingivalis gene for 16S rRNA, partial sequence, strain:ATCC33277	32	16	4.7	AB035459
Porphyromonas gingivalis gene for 16S rRNA, partial sequence, strain:A7A1-28	32	16	4.7	AB035458

Sequence	Score	Length	Expect	Accession Number
Porphyromonas gingivalis gene for 16S rRNA, partial sequence, strain:SUNY1021	32	16	4.7	AB035457
Porphyromonas gingivalis gene for 16S rRNA, partial sequence, strain:W83	32	16	4.7	AB035456
Porphyromonas gingivalis gene for 16S rRNA, partial sequence, strain:FDC 381	32	16	4.7	AB035455
Unidentified rumen bacterium RFN46 gene for 16S ribosomal RNA, partial sequence	32	16	4.7	AB009202

However, even in the unlikely event that the forward and reverse primers used in an FMDV TaqMan™ assay amplify one of these other sequences, the homology between a polynucleotide having the sequence of SEQ ID NO:1 and these other sequences is not great enough to result in the identification of a false positive using the TaqMan™ assay.

The skilled person will appreciate that the sequence of SEQ ID NO:1 can be modified, although such modifications are functionally constrained. Preferably TaqMan™ probes should preferably not have a G at the 5'-end, should not have more Gs than Cs and should not have more than 4 Gs in a row. Typically a TaqMan™ probe should have a higher melting temperature than the primers it is used in conjunction with. Software suitable for designing TaqMan™ probes includes Primer Express available from PE Applied Biosystems, Warrington, Cheshire, UK.

If modifications are made to the probe, such as deletions, the modified probe (eg, the fragment) should be capable of binding specifically to the complementary sequences of SEQ ID NO: 1. By "capable of binding

specifically to the complementary sequence of SEQ ID NO: 1 " is included the meaning that, in a TaqMan™ assay, the probe will bind to and be degraded during the amplification of a sequence comprising the complementary sequence of SEQ ID NO: 1 but will show substantially reduced binding and degradation, such as typically at most 75%, preferably at most 50%, more preferably at most 25%, yet more preferably at most 10%, even more preferably at most 5% degradation (as a percentage of the degradation shown under the same conditions during the amplification of a sequence comprising the complementary sequence of SEQ ID NO: 1) during the amplification of a sequence comprising the complement of a sequence having up to 18 consecutive or 19 out of 20 bases identical to SEQ ID NO: 1. In other words a modified probe must be capable of distinguishing between FMDV derived polynucleotide and a contaminating highly homologous polynucleotide.

Typically a modified probe will retain the melting temperature of a polynucleotide having the sequence defined in SEQ ID NO: 1. For example, it is possible to generate fragments of the probe. Such fragments may have 1, 2, 3, 4 or 5 nucleotide deletions. Preferably the deletions are of bases adjacent to one another. Preferably the deletions are made from either or both of the 5' or 3' ends of the probe. When the probe is shortened by deletion, synthetic nucleotide bases can be incorporated into the probe to maintain the melting temperature of the modified probe at substantially the same melting temperature as the unmodified probe defined as SEQ ID NO:1. Synthetic nucleotides are known in the art and are available commercially (eg, from PE Applied Biosystems, Warrington, Cheshire, UK). Where a synthetic nucleotide is incorporated in a probe it is preferably positioned in the unique region of the probe, especially when that synthetic nucleotide is intended to act to retain the melting temperature of the unmodified probe. The term "unique region" refers to the region of the

probe that is divergent from the sequences of any homologous genes not found in the FMDV genome.

By "substantially the same melting temperature" is meant that the melting temperature is modified by at most 5°C, preferably by at most 2°, more preferably by at most 1°C, even more preferably by at most 0.5°C, yet more preferably by at most 0.1°C, most preferably there will be substantially no difference in the melting temperatures of the modified and unmodified probes. In practice, the modified probe must be able to operate in a TaqManTM assay to discriminate between FMDV polynucleotide and other highly homologous but different polynucleotides that may have up to 18 consecutive or 19 out 20 identical bases to the probe binding site in the FMDV genome.

The polynucleotide of the first aspect of the invention may comprise a labelling moiety. In a preferred embodiment of the first aspect of the invention the polynucleotide comprises two labelling moieties. Any labelling moiety may be used. In a preferred embodiment, the or each moiety is capable of acting as a fluorescent marker. A moiety is capable of acting as a fluorescent marker if it can receive radiation at one wavelength (the excitatory wavelength) and, as a result, emit radiation at a different wavelength. Particularly preferred fluorescent markers include FAM (6-carboxy-fluorescein), TAMRA (6-carboxy-tetramethyl-rhodamine), TET (tetrachloro-6-carboxy-fluorescein) and HEX (hexachloro-6-carboxy-fluorescein).

Where a polynucleotide of the first aspect of the invention comprises two fluorescent markers it is preferred that the probe is designed so that one moiety (the quencher) can quench the fluorescence emission of the other moiety (the reporter). It is even more preferred that the moieties are bound

to the polynucleotide at positions that allow the quencher to quench the fluorescence emission of the reporter.

By "one moiety can quench the fluorescence emission of the other moiety" is included the meaning that, in the intact probe, at least 10%, typically at least 20%, 30% or 40%, usually at least 50%, preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, yet more preferably at least 90%, most preferably substantially 100% of the fluorescence emissions from the reporter are absorbed by the quencher. Usually quenching will occur under conditions used during RT-PCR.

Thus, the reporter absorbs radiation at one wavelength (1) and emits it at a different wavelength (2). Where the reporter is present alone, the only detectable fluorescence in response to the provision of radiation at wavelength (1) will be emissions at wavelength (2).

However, the quencher can absorb radiation at wavelength (2) (ie, the same wavelength as emitted by the reporter) and emit it at yet another wavelength (3). Accordingly, where both the reporter and quencher are present, provision of radiation at wavelength (1) can result in production of fluorescence emissions at both wavelengths (2) and (3), or even (3) only. The ratio between (2) and (3) is dependent on the percentage of fluorescence emissions (2) from the reporter absorbed by the quencher.

One factor in determining ratio between (2) and (3) is the spatial relationship between the first moiety (reporter) and second moiety (quencher) second moieties. The closer the moieties are to one another, the greater the percentage of fluorescence emissions (2) from a first moiety absorbed by a second moiety will be.

Thus changes in the spatial relationship between the first and second moieties can be determined by monitoring changes in the ratio between the fluorescence emissions of the first (2) and second (3) moieties in response to the input of radiation at the excitatory wavelength of the first moiety (1).

By "bound to the polynucleotide at positions that allow one moiety to quench the fluorescence emission of the other moiety" is included the meaning that the spatial relationship between the first (reporter) and the second (quencher) moiety is such that "one moiety can quench the fluorescence emission of the other moiety" as defined above. Typically the nucleotide through which the first moiety is attached to the polynucleotide of the first aspect of the invention is not the same as the nucleotide through which the second moiety is attached. Usually the positions of the nucleotides through which the first and the second moieties are attached to the polynucleotide of the first aspect of the invention are different by up to 1, 2, 7, 11, 15, 19 or 23 positions. Thus where the positions of the nucleotides through which the first and the second moieties are attached to the polynucleotide of the first aspect of the invention are different by 23 positions, the moieties will be attached by nucleotides 1 and 24, ie, the 5' and 3' terminal nucleotides. Where the positions of the nucleotides through which the first and the second moieties are attached to the unmodified polynucleotide of the first aspect of the invention are different by less than 23 positions, then either one, the other, or neither of the positions of said nucleotides will be the 5' and 3' terminal nucleotides. It is preferred that at least one of the moieties will be attached via a terminal nucleotide, most preferably via the terminal nucleotide in the 5' end.

Thus, where polynucleotides of the first aspect of the invention comprise two moieties, one of which (the second) can quench the fluorescence emissions of the other (the first), it is possible to measure the degradation of

that polynucleotide by following changes in the ratio between emissions of the first (2) and second (3) moieties over time in response to provision of radiation at the excitatory wavelength of the first moiety (1). As the polynucleotide is degraded, the physical connection between first and second moiety is broken and the spatial relationship altered, ie, the second (quencher) moiety is separated from the first (reporter) moiety. Separation is indicated by a reduction in detectable fluorescence emissions of the second (quencher) moiety (3) and a concomitant increase in the detectable fluorescence emissions of the first (reporter) moiety (2).

Thus, where the polynucleotide of the first aspect of the invention is used as a probe in a TaqMan™ assay, it will typically comprise two moieties, the identities of, and the spatial relationship between, the moieties being selected so that one moiety can quench the fluorescence emission of the other moiety. Typically, if the first moiety is FAM, TET or HEX, then the second moiety will be TAMRA, although the skilled person will understand that any combination of moieties can be used so long as a detectable quenching of the fluorescence emissions of one moiety (the reporter) by another moiety (the quencher) occurs in the intact probe.

In a second aspect of the invention, there is provided an isolated polynucleotide having the sequence of SEQ ID NO:2:

SEQ ID NO:2

CAC{T/C}T{T/C}AAG{G/A}TGACA{T/C}TG{G/A}TACTGGTAC.

The degeneracy of the polynucleotide is represented by alternative nucleotides presented in parenthesis. For example, "{A/G}" means that, at that position, the polynucleotide is degenerate and a proportion of the population of polynucleotide molecules will contain an A at that position

whereas a proportion of the population of polynucleotide molecules will contain a G at that position. Typically the proportion of molecules having one particular nucleotide at a degenerate position compared to the proportion of molecules having another particular nucleotide at that position is substantially 50/50, although it is possible to weight the distribution one way or the other, such as about 95/5, 90/10, 80/20, 70/30, 60/40, 40/60, 30/70, 20/80, 10/90 or 5/95.

Thus, SEQ ID NO:2 denotes a population of polynucleotides having one of 32 possible nucleotide sequences. Preferably, there is provided a population of polynucleotides wherein the population comprises polynucleotides having at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 or 30 of the 32 possible nucleotide sequences denoted by SEQ ID NO:2. More preferably, the population comprises polynucleotides having each of the 32 possible nucleotide sequences denoted by SEQ ID NO:2.

In a third aspect of the invention, there is provided an isolated polynucleotide having the sequence of SEQ ID NO:3.

SEQ ID NO: 3

CAGAT{C/T}CC{G/A}AGTG{T/A}C{I}C{I}TGTTA

The degeneracy of the polynucleotide is represented in the same way as described above in respect of SEQ ID NO: 2. "{I}" refers to the inclusion of inosine as a non-specific base at the defined position. Inclusion of inosine is a further means of producing a degenerate polynucleotide and is equivalent to providing polynucleotides having each of the four conventional bases (*i.e.* A, C, T and G) at the defined position. It will be appreciated by persons skilled in the art that one or both of the inosine bases

may be replaced with a population of polynucleotides having A, C, T or G at the equivalent position.

Thus, as in the case of SEQ ID NO:2 (see above), SEQ ID NO:3 denotes a population of polynucleotides having one of 8 possible nucleotide sequences (where both inosine bases are replaced by polynucleotides having A, C, T and G at the equivalent positions, there are 128 possible sequences). Preferably, there is provided a population of polynucleotides wherein the population comprises polynucleotides having at least 2, 3, 4, 5, 6 or 7 of the 8 possible nucleotide sequences denoted by SEQ ID NO:3. More preferably, the population comprises polynucleotides having each of the possible nucleotide sequences denoted by SEQ ID NO:3.

The polynucleotides of the second and third aspects of the invention have been designed to act as 'universal' primers for all FMDV strains. They can be used together to prime the PCR amplification of cDNA generated from the FMDV genome. The resultant PCR product contains a sequence complementary to the sequence defined by SEQ ID NO: 1.

The skilled person will appreciate that other primers may be used for this task, either in place of one of both of the polynucleotides of the second and third aspects of the invention. Typically primers are designed so that the resultant PCR product is no more that 10kb, preferably no more that 2kb, more preferably no more than 1kb, even more preferably no more than 0.5kb, yet more preferably no more than 0.2 kb, most preferably no more than 0.1kb in length. The size of the PCR product is determined by the distance between the primer annealing sites in the target polynucleotide. The primers should be designed to flank the region of cDNA generated from the FMDV genome that contains the sequence complementary to the

SEQ ID NO: 1 (the probe binding site). Any primer size may be used, although suitable primers are typically about 15-25 nucleotides in length.

Suitable primer annealing sites can be chosen based on their positions relative to the probe binding site and each other. Other criteria include the relative homology in the annealing site between different FMDV strains. This can be determined by aligning sequences of different FMDV strains 5'and 3' to the probe binding site, for instance using publicly available computer software (see below). Suitable primer sequences will typically have at least 50%, preferably 60%, more preferably 70%, yet more preferably 80%, even more preferably 90%, most preferably 95% or more sequence homology between the different FMDV strains. Higher percentages of homology are preferred because it reduces the number of degenerate nucleotides required to produce a 'universal' primer, thus improving the specificity of PCR amplification performed using the primers. "Percent (%) sequence homology" is defined as the percentage of nucleic acid residues in a candidate primer sequence that are identical with the nucleic acid residues of the sequences of interest of the different FMDV strains, after aligning the sequences and introducing gaps, if necessary to achieve maximum percent sequence homology, and not considering any conservative substitutions as part of the sequence homology. Methods for performing sequence alignment and determining sequence homology are known in the art, may be performed without undue experimentation, and calculations of % homology values may be obtained for example, using available computer programs such as WU-BLAST-2 (Altschul *et al*, 1996, *Methods in Enzymology* **266**,460-480) or other commercially available programs like Omiga from Oxford Molecular/Pharmacopoeia. One may optionally perform the alignment using set default parameters in the computer software program (Blast search, MacVector and Vector NTI).

Polynucleotides of the first, second and third aspects of the invention can be produced by any suitable method. Conveniently they will be produced by synthetic methods well known in the art, usually utilising a solid-support such as controlled-pore glass or polystyrene (for a review see Sambrook and Russell (2001) *Molecular Cloning, A Laboratory Manual*, (3rd Ed) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY and the references therein). Automated synthetic machines are available for this purpose and custom-synthesised polynucleotides of defined sequence can be obtained commercially from a number of companies (such as PE Applied Biosystems, Warrington, Cheshire, UK).

In a fourth aspect of the invention, there is provided a method for determining whether a sample contains FMDV comprising:

(a) contacting the sample with a probe comprising a polynucleotide according to the first aspect of the invention *in vitro* under conditions that allow the probe to bind specifically to a target polynucleotide; and

(b) determining whether the probe has bound to a target polynucleotide;

thereby to determine whether the sample comprises a target polynucleotide, the presence of a target polynucleotide in a sample being indicative of presence of FMDV.

The term "sample" as used herein includes the meaning of any polynucleotide, typically RNA, cDNA or DNA, derivable directly or indirectly from matter of interest. The term "matter of interest" includes, but is not limited to substances taken from or produced by the body of an animal, air or water taken from the environment, sewage effluent or foodstuffs.

In a preferred embodiment of the fourth aspect of the invention, the sample has been taken from a mammal. Preferably the mammal is non-human. Usually the mammal is a domestic or wild cloven-hoofed animal, such as a pig, or a wild or domesticated ruminant, such as cattle, a buffalo, a sheep, a goat or a deer. Advantageously, the sample is a blood sample or oesophageal-pharyngeal ('probang') sample.

Samples for use in a method according to the fourth aspect of the invention may be in any form. When they are taken from the body of an animal, the sample is preferably *ex vivo*. This will typically be the case as body samples will be taken remotely and transferred to a testing facility for assay. The skilled person will appreciate that any type of sample may be used. Commonly, samples are epithelial lesions. Other suitable samples include blood samples, tissues, swabs, probangs (collection of fluid from the throat). The assay will thus be performed on an *ex vivo* sample.

The sample may be prepared for use in a method according to the fourth aspect of the invention. Typically preparation of the sample includes isolation of FMDV RNA from the sample. Alternatively preparation of the sample includes total nucleic acid extraction from the sample. Techniques for RNA isolations and total nucleic acid extraction are well known in the art (Sambrook and Russell (2001) *Molecular Cloning, A Laboratory Manual*, (3rd Ed) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

In a preferred embodiment of the fourth aspect of the invention, the isolated FMDV RNA is reverse transcribed using a suitable enzyme such as a retroviral reverse transcriptase or commercially available variant thereof (Sambrook and Russell (2001) *Molecular Cloning, A Laboratory Manual*, (3rd Ed) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Reverse transcription may be primed by any method known in the art, such as by using specific primers to reverse transcribe selected regions of the FMDV genome. However, bearing in mind the high level of variability exhibited between different FMDV strains, random priming using, for example random hexamers, is usually suitable. Commercially available kits, such as the TaqMan™ RT kit (PE Applied Biosystems, Warrington, Cheshire, UK), are generally suitable for this purpose. Reverse transcription of the RNA produces complementary DNA (cDNA).

The method of the fourth aspect of the invention may be used for determining whether a sample contains FMDV. Therefore, the method of the fourth aspect of the invention is able to indicate whether or not FMDV genomic RNA was present in the sample. In other words the method of the fourth aspect of the invention provides a qualitative assay for FMDV. In a preferred embodiment the method will be able to positively identify a sample that contains as few as 1000, more preferably 100, yet more preferably 10 FMDV genomes.

The method of the fourth aspect of the invention may further provide a quantitative assay for FMDV. Accordingly it is possible to determine the relative abundance of FMDV in a sample compared to, eg, a control of known FMDV abundance and/or in comparison to other samples. Where the method of the fourth aspect of the invention is quantitative, this will allow the skilled person to determine, for example, whether virus levels are increasing or decreasing over time in the matter of interest. Quantitative assays are well known in the art (Sambrook and Russell (2001) *Molecular Cloning, A Laboratory Manual*, (3rd Ed) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and can be performed, for example, using a TaqMan™ assay (see below).

The method of the fourth aspect of the invention comprises contacting the sample with a probe comprising a polynucleotide according to the first aspect of the invention *in vitro* under conditions that allow the probe to bind specifically to a target polynucleotide. By "conditions that allow the probe to bind specifically to a target polynucleotide" is included any conditions under which the probe will display detectable binding to the target consensus sequence of the target polynucleotide derived from the FMDV genome (such as RNA or, preferably, cDNA) whilst displaying substantially reduced, or preferably undetectable binding to other polynucleotides having sequences with up to 18 consecutive or 19 out 20 bases identical to the target consensus sequence. Such conditions are well known in the art, for example, conditions used in the exemplified TaqMan™ assay below. The suitability of other conditions may, for example, be tested by amplifying a PCR product that has up to 18 consecutive or 19 out of 20 bases identical to the target consensus sequence and determining whether probe degradation occurs. If substantially no probe binding occurs in that sample, but FMDV can be detected using the same conditions, then those conditions are suitable to allow the probe to bind specifically to a target polynucleotide.

The method of the fourth aspect of the invention also comprises determining whether the probe has bound to a target polynucleotide. Numerous methods are available in the prior art for determining whether a probe has bound to a target polynucleotide (Sambrook and Russell (2001) *Molecular Cloning, A Laboratory Manual*, (3rd Ed) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and the skilled person will appreciate that any suitable detection method known in the art can be employed to determine whether the probe has bound to a target polynucleotide.

In a preferred embodiment of the fourth aspect of the invention, the step of determining whether the probe has bound to a target polynucleotide comprises amplifying a region of the target polynucleotide, which region comprises the binding site of the probe. Typically amplification will be performed by PCR, although the skilled person will appreciate that other methods of amplification may be used. The PCR amplification may be primed by either, both, or neither of the polynucleotides according to the second and third aspects of the invention. Where one or both of the polynucleotides according to the second and third aspects of the invention are not used in PCR amplification, they may be replaced with alternative primers, typically designed in accordance with the guidance provided above.

In a particularly preferred embodiment, the method of the fourth aspect of the invention utilises a TaqMan™ assay. Accordingly step (a) comprises contacting FMDV cDNA derived from the sample with the probe polynucleotide of the first aspect of the invention. Step (b) comprises of determining whether the probe has bound to a target polynucleotide and is achieved using a TaqMan™ assay. PCR amplification primed by suitable primers, such as the polynucleotides defined in the second and third aspects of the invention, results in the degradation of the probe polynucleotide as defined in the first aspect of the invention by the 5'-3' exonuclease activity of the Taq DNA polymerase used. Degradation of the polynucleotide probe according to the first aspect of the invention can then be determined.

Typically the polynucleotide probe of the first aspect of the invention will comprise two moieties, one of which (the quencher) can quench the fluorescence emission of the other (the reporter), which moieties are bound to the polynucleotide at positions that allow one moiety (the quencher) to quench the fluorescence emission of the other moiety (the reporter).

Accordingly, the degradation of the polynucleotide probe according to the first aspect of the invention can be measured by measuring quenching at each cycle of the PCR amplification. If a cDNA having a primer and probe binding sites (ie, FMDV cDNA) is present in the sample, then the probe will bind to the cDNA during the annealing step of each PCR cycle and be degraded by the Taq DNA polymerase during the elongation step of each cycle.

Thus samples comprising FMDV cDNA will show reduced quenching with each subsequent round of PCR amplification, whereas the quenching in samples not containing FMDV cDNA will remain substantially unchanged. Thus the TaqMan™ assay provides a qualitative assay for the presence or absence of FMDV in a sample. Moreover, since the amount of quenching is dependent on the amount of FMDV cDNA target initially present in a sample, it is possible to use the TaqMan™ assay to compare the abundance of FMDV in one sample with another sample and/or a control having a known abundance of FMDV. In other words the TaqMan™ assay may also be used as a quantitative assay.

Thus, in a preferred embodiment of the fourth aspect of the invention, the step of determining whether the probe has bound to a target polynucleotide comprises determining the fluorescence emissions of the probe.

Numerous methods are known in the art for determining fluorescence emissions. Where the assay method involves a TaqMan™ assay, conveniently probe quenching can be determined at each round of amplification using suitable equipment such as PE Applied Biosystems (Warrington, Cheshire, UK) models 5700 or 7700. Alternatively, quenching can be determined after completion of PCR amplification. However, determination of probe quenching during amplification is

preferred because the shape of the amplification plot can be utilised to separate false positives from true positives.

In a preferred embodiment, the method of the fourth aspect of the invention is automated. By 'automated' we mean that one or more of the steps of the method are performed by a machine. Exemplary protocols and apparatus for performing such automated methods are described in Examples 2 and 3 below.

The skilled person will appreciate that there are many applications for the method according to the fourth aspect of the invention. The following examples are merely illustrative and non-limiting on the potential uses of the method of the fourth aspect of the invention.

The invention provides a method for determining whether an organism is infected with FMDV comprising determining whether a sample, preferably on the *ex vivo* sample, from the organism contains FMDV by a method according to the fourth embodiment of the invention.

The invention provides a method of vaccinating an organism against FMDV comprising determining whether a sample from an organism contains FMDV by a method according to the fourth embodiment of the invention, and, if FMDV is not detected in the sample, administering an FMDV vaccine to the organism. Vaccines for FMDV are known in the art and include inactivated whole viruses, and are available commercially from companies such as Merial, Bayer and Intervet, as well as vaccines described in US 6,107,021 and US 4,732,971. Generally, primary administration of an FMDV vaccine is followed with a booster, for example, after 1 month, which may be followed by a further regular boosters, for example, every 6 months.

The invention provides a method of treating an organism infected with FMDV comprising determining whether a sample from an organism contains FMDV by a method according to the fourth embodiment of the invention, and, if FMDV is detected in the sample, administering a therapeutic agent to the organism, which agent is effective in combating the FMDV virus. By "treating" is included the meaning that one or more symptoms of FMD are ameliorated. Preferably treatment includes providing relief from pain. Treatment may include substantially reversing the effects of FMD on production losses. Any therapeutic agent, such as interferon-gamma, may be used. A therapeutic agent is "effective in combating the FMDV virus" if it is capable of treating FMDV as defined above.

The form, amount and administrative regime used for treatment of FMDV with a therapeutic agent will be apparent to the skilled person and will be typically determined based on the identity, age, weight and/or condition of the recipient. A therapeutic agent or a formulation thereof may be administered by any conventional method including oral and parenteral (eg subcutaneous or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time. Whilst it is possible for a therapeutic agent to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the therapeutic agent and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

The invention provides a method for combating the spread of FMDV between organisms comprising determining whether a sample from an

organism contains FMDV by a method according to the fourth embodiment of the invention, and, if the organism is infected with FMDV sacrificing the organism. By "combating the spread of FMDV between organisms" is meant that the rate of spread of FMDV is reduced in comparison to an analogous situation where no action is taken. After the organism is sacrificed, typically the corpse will be disposed of, for example by cremation or burial, to prevent or reduce the spread of FMDV from the corpse.

The invention provides a method for determining whether a test vaccine is capable of preventing FMDV infection comprising administering the test vaccine to an organism, inoculating the organism with FMDV and determining whether a sample from the organism contains FMDV by a method according to the fourth embodiment of the invention. Any molecule or combination thereof can be used as a test vaccine. Typically the test vaccine may comprise a polypeptide or polynucleotide derived from FMDV, or a synthetically modified version thereof. The vaccine can be administered to the recipient is any suitable form. Typically the recipient is maintained for a period of time, such as one week, one months or six months, before inoculation, in order to allow the test vaccine to mediate an immune response in the recipient, and thus immunise the recipient against FMDV infection. Further administrations of the test vaccine may be made during the maintenance period. Inoculation with FMDV by techniques known in the art (Burrows, *J Hyg (Lond)*, 1966, **64**:419-429; Burrows, *J Hyg (Lond)*, 1968, **66**:633-640; Henderson, *J Hyg (Lond)*, 1952, **50**:182-194; Sellers *et al*, *Vet Rec*, 1969, **85**:198-199) typically follows. After a brief period of time to allow the establishment of the FMD, samples may then be taken from the recipient organism, typically at regular intervals, such as daily during the first and typically also the second week post inoculation and, thereafter, weekly. The presence (or absence) of, and

levels of, FMDV in the samples can then be determined using a method according to the fourth aspect of the invention in order to trace the progress of the disease, thereby to determine whether the test vaccine has been able to render the recipient organism immune to FMDV.

The invention provides a method for determining whether a test agent is capable of combating FMDV comprising administering the test agent to an organism infected with FMDV and determining whether the test agent is capable of combating FMDV by a method according to the fourth embodiment of the invention. Inoculation with FMDV can be achieved by techniques known in the art (Burrows, *J Hyg (Lond)*, 1966, **64**:419-429; Burrows, *J Hyg (Lond)*, 1968, **66**:633-640; Henderson, *J Hyg (Lond)*, 1952, **50**:182-194; Sellers *et al*, *Vet Rec*, 1969, **85**:198-199). Typically the recipient is maintained for a period of time, such as up to one day, one week, one month or six months before administration of the test agent begins. Any molecule or combination thereof can be used as a test agent. The test agent can be administered to the recipient in any suitable form, any suitable dosage and using any suitable regimen. This can be determined by the skilled person on the basis of identity, age, weight and condition of the recipient. However, with experimental test agents, initial empirical testing may additionally be required to determine toxicity levels and the like prior to attempts to treat FMDV. A sample, typically a plurality of samples, are taken from the recipient organism. At least one sample may be taken after inoculation of the recipient but before onset of administration of the test agent to provide a 'pre-treatment' reading. The presence of, and levels of, FMDV in the samples can then be determined using a method according to the fourth aspect of the invention in order to trace the progress of the disease, thereby to determine whether the test agent has been able to combat the proliferation of FMDV in the recipient organism.

In a fifth aspect of the invention, there is provided the use of a polynucleotide as defined in the first, second or third aspect of the invention for detecting FMDV. A polynucleotide as defined in the first, second or third aspect of the invention may be used for detecting FMDV, for example, using any of the methods described above.

In a sixth aspect of the invention, there is provided a system for detecting FMDV comprising a polynucleotide as defined in the first aspect of the invention, wherein the specific binding of the polynucleotide to a target polynucleotide of a sample is indicative of the presence of FMDV in the sample.

In a seventh aspect of the invention, there is provided a kit of parts comprising a polynucleotide as defined in the first aspect of the invention.

Optionally the kit is further provided with a polynucleotide as defined in the second aspect of the invention and/or a polynucleotide as defined in the third aspect of the invention. The skilled person will appreciate that either or both of the polynucleotides of the second and third aspects of the invention can be replaced by alternative polynucleotide primers designed in accordance with the guidelines given above.

A kit according to the seventh aspect of the invention may additionally include a means of polynucleotide isolation. It may include associated equipment such as a centrifuge, means for polynucleotide quantitation such as a spectrophotometer. It may include means for reverse transcription of RNA to cDNA such as the TaqMan™ RT kit (PE Applied Biosystems, Warrington, Cheshire, UK). It may include means for performing a PCR reaction such as PE Applied Biosystems (Warrington, Cheshire, UK) models 5700 or 7700 which are capable of performing the PCR reaction

using a thermal cycling function and simultaneously measuring probe quenching at each round of amplification. However, many or all of these items will be readily available in a molecular biology laboratory. Therefore, a kit according to the seventh aspect of the invention may additionally comprise dNTPs suitable for dilution to a convenient concentration for use in reverse transcription and PCR. It may include suitable buffers for performing polynucleotide isolation, reverse transcription or PCR amplification. In particular, it may include a buffer that provides conditions that allow the probe polynucleotide according to the first aspect of the invention to bind specifically to a target polynucleotide. For example, a total volume of 24 µl of TaqMan™ PCR mix, may contain 0.3-0.9 pmol/µl of forward primer, 0.9 pmol/µl of reverse primer, 0.2 pmol/µl of TaqMan™ probe and 1 X TaqMan™ master mix (Applied Biosystems) which may, for example, be added to the well of a 96-well optical plate (Applied Biosystems) and followed by 1 µl of cDNA. The kit may further comprise instructions for using the components and may therefore include instructions for performance of the PCR cycle. A PCR typical cycle is 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 sec, 60°C for 1 min, 50 cycles.

A kit of the invention may further comprise polynucleotide samples having known concentrations of FMDV RNA or cDNA. Such samples may, for example, be used as controls or standards against which test samples can be adjudged. A kit of the invention may further comprise primers and probe for use as an internal control. Suitable positive controls typically comprise field material, cell cultured material or material collected from experimentally infected animals, containing known amounts of FMDV. Negative controls may be provided and typically comprise negative samples from tissues or cell culture of the same type as the positive samples. Controls may be treated, for example with Trizol (Life Technologies), Lysis

solution (Roche) or RNAlater (Ambion), in order to stabilise the RNA contents. Suitable controls may also be prepared by *in vitro* transcription of RNA, usually from cloned plasmids, containing the region of interest. In a preferred embodiment of the seventh aspect of the invention, a control may be manufactured using a cloned polynucleotide fragment (such as a DNA) comprising the FMDV area in question by conversion into 'armoured' RNA, by using technology available from Ambion.

The invention will now be described in more detail by reference to the following Figures and Examples wherein:

Figure 1 shows the sigmoidal amplification plot of a positive sample for FMD virus by real-time, fluorogenic RT-PCR assay. The threshold line is set at 0.500 and the C_T value (where the plot intercepts the threshold line) is 23.00.

Figure 2 shows a non-sigmoidal amplification plot of a sample. The fluorogenic RT-PCR assay of the sample should be repeated.

Figure 3 shows (a) a scatter diagram of the Ct values obtained by the 32-well RT-PCR using automated programmes for nucleic acid extraction and reverse transcription (manual pipetting for PCR amplification) on epithelial suspensions of field samples, (b) a scatter diagram of the Ct values obtained by the 32-well fully automated RT-PCR procedure on epithelial supensions of field samples and (c) a scatter diagram of the Ct values obtained by the 96-well 'fast RT-PCR protocol' on epithelial suspensions of field samples.

Figure 4 shows an optics graph of PCR of diluted plasmid DNA performed on the Cephoid SmartCycler real-time PCR machine using core reagents and PCR beads. Serial dilutions from 10^{-5} to 10^{-9} and a water-only negative

control were amplified via both assays. Samples which tested negative are not labelled individually but appear as a flat line with zero fluorescence.

Figure 5 shows gel electrophoresis image of PCR products of diluted plasmid DNA using core reagents and PCR beads analysed on a 1.5% agarose gel run for approximately 1 hour at 100V.

Figure 6 shows graphs comparing average results obtained for each of the 3 different PCR assays from the analysis of nasal swab samples from the 4 contact (A) and 6 inoculated (B) animals over a 10 day period. The reaction of the samples are given as 50-Ct (a total of 50 cycles done minus the threshold value) in order to make visual examination easier, as strong positive samples are shown as a high value and weak samples shown as a low value or zero (*i.e.* a Ct value of 50).

EXAMPLE

Example 1: Detection of all seven serotypes of Foot-and-Mouth Disease virus by real-time, fluorogenic RT-PCR assay

Materials and methods

Sample preparation

Epithelial suspensions (ES) of reference samples of all seven FMD virus serotypes as well as swine vesicular disease (SVD) virus were prepared in phosphate buffer (Ferris and Dawson, 1988) at the time of sample receipt. In some cases, vesicular fluid or cell culture material was submitted for diagnosis in the absence of vesicular epithelium. Cell culture grown antigens resulting from the inoculation of ES or original material onto primary calf thyroid or kidney cell cultures or a permanent cell line of porcine kidney cells (IB-RS-2) were also prepared at the time of receipt. Fresh ES and cell culture grown isolates of some reference samples were prepared as described (Ferris and Dawson, 1988) since insufficient volumes of the original ES and cell culture virus preparations were available for testing. Fresh ES and calf thyroid cell culture virus preparations were similarly prepared from samples submitted to the WRL for FMD for virus diagnosis during the UK 2001 epidemic. Cell culture supernatant fluids were prepared after inoculation of oesophageal-pharyngeal fluids ("probangs") onto calf thyroid cell cultures and supernatant fluids from cells infected with vesicular stomatitis (VS) virus and vesivirus (including San Miguel sea lion virus), which also cause vesicular diseases, were prepared as above to test the specificity of the fluorogenic and conventional RT-PCR methods for detection of FMD viral genome. Suspensions prepared from normal bovine epithelium and from uninoculated primary calf thyroid and

IB-RS-2 cell culture supernatant fluid were used as negative controls. The reference samples tested by the fluorogenic and conventional RT-PCR methods are listed in Table 1. Samples submitted from the UK 2001 FMD epidemic are listed in Tables 1 and 3.

RNA extraction

Total RNA was extracted from each sample with TRIzol® Reagent (Life Technologies, UK) as described previously (Reid et al., 1998; 1999; 2000). The same batch of extracted RNA was used when samples were tested both in the fluorogenic and conventional RT-PCR procedures.

Reverse transcription

RNA was subjected to reverse transcription using random primers at 37°C for 45 min in a 20 µl reaction volume as described previously (Reid et al., 1999). The shorter protocol described by Reid et al. (Reid et al., 1999), in which samples were subjected to reverse transcription at 37°C for 15 min, was used on a small selection of the RNA extracted from FMD viruses of the SAT serotypes.

Primer/probe design

(i) Fluorogenic RT-PCR

A TaqMan® probe, SAmulti2-P-IR-292-269R of 24 nucleotides (SEQ ID NO. 1: 5' - CCT CGG GGT ACC TGA AGG GCA TCC - 3') was designed using Primer Express Software (Applied Biosystems, UK) from internal ribosomal entry site (IRES) sequences (within the FMD virus RNA 5'-untranslated region) available from public databases and unique, non-public

sequence data from the Institute for Animal Health, Pirbright. The probe sequence is totally conserved in and specific to FMD viruses; making this genomic region highly suitable for the detection of all seven serotypes of FMD virus. However, there is some homology with certain other cellular genes which likely stem from the function of this region but the maximum identity between the probe sequence region and cellular sequences was a single stretch of 18 nucleotides or, in one case, 19 out of 20 nucleotides. At the temperature chosen for the PCR amplification, this degree of homology is not sufficient to give a background signal. Redundant primers SA-IR-219-246F (SEQ ID NO. 2: 5' - CAC YTY AAG RTG ACA YTG RTA CTG GTA C - 3') and SA-IR-315-293R (SEQ ID NO. 3: 5' - CAG ATY CCR AGT GWC ICI TGT TA - 3') on each side of the conserved probe were designed using the Primer Express Software and aligned sequences from public databases and unique, non-public sequence data from the Institute for Animal Health, Pirbright, to enable the assay to detect all seven serotypes of FMD virus. Amplicon size was short (c120 nucleotides) which enhanced the PCR efficiency and lessened the risk of any RNA degradation affecting successful amplification.

(ii) Conventional RT-PCR

Two sets of primers were used in the conventional RT-PCR. One set, O/A/C/Asia 1 (1F/1R), was designed for the universal diagnosis of all seven serotypes of FMD virus (Reid et al, 2000). The other set (O-type specific O1Kauf3800/P2) was designed for the specific detection of FMD virus serotype O (Alexandersen et al., 2000), and its sequence and genomic location are shown in Table 4. The expected amplicon sizes of the 1F/1R and O-type specific O1Kauf3800/P2 primer sets were 328 bp and 150 bp respectively. Primers 1F, 1R and P2 were made by Cruachem Ltd. (UK) and the O-type specific O1Kauf3800 primer made by MWG Biotech AG (UK).

Fluorogenic RT-PCR

The fluorogenic RT-PCR procedure was similar to that described by Oleksiewicz et al. (2001). For the testing of reference FMD viruses and the other vesicular disease viruses, PCR mixes containing 0.3 pmol/µl of forward primer SA-IR-219-246F, 0.9 pmol/µl of reverse primer SA-IR-315-293R, 0.2 pmol/µl of probe SAmulti2-P-IR-292-269R and 1x TaqMan® master mix (Applied Biosystems) were made up in either nuclease-free water (Promega) or Milli-Q water (Millipore, UK) and 24 µl of the mix added to a MicroAmp® optical 96-well reaction plate (Applied Biosystems) followed by 1 µl of cDNA. The same procedure was used to test the samples submitted from the UK 2001 FMD outbreak except that 0.9 pmol/µl of both the forward and reverse primer were used in each PCR reaction. Whenever possible, cDNA samples of reference viruses or UK sample submissions were tested in duplicate to assess the intra-assay reproducibility and several cDNA samples were tested on more than one occasion to assess inter-assay reproducibility. Amplification was carried out in a GeneAmp® 5700 Sequence Detection System thermal cycler (Applied Biosystems) using the following programme: 50°C for 2 min (uracil N-deglycosylase digest), 1 cycle; 95°C for 10 min (activation of the Taq Gold thermostable DNA polymerase present in the master mix), 1 cycle; 95°C for 15 sec, 60°C for 1 min; 50 cycles. After amplification, a threshold cycle (CT) value was assigned to each PCR reaction as described previously (Oleksiewicz et al., 2001). The absolute negative value for any test sample or negative control corresponded to a CT value of 50.00.

Conventional RT-PCR

Reference samples were tested by the conventional RT-PCR procedure with the universal O/A/C/Asia (1F/1R) primer set (Reid et al., 2000). Samples submitted from the UK FMD outbreak in 2001 were tested by the same RT-PCR procedure but using the O-type specific O1Kauf3800/P2 primer set (Alexandersen et al., 2000) as a strain of FMD virus serotype O was the causative agent of the UK 2001 outbreak.

ELISA

The FMD virus serotype of the ES prepared from the reference samples and the supernatant fluids resulting from their passage in cell cultures was confirmed by ELISA (Ferris and Dawson, 1988) at the time of sample receipt or at the time of sample preparation (data not shown) as was the FMD virus specificity of the ES and cell culture supernatant fluids prepared from the UK 2001 sample submissions. The UK sample submissions testing positive by ELISA on ES, confirmed positive by virus isolation in calf thyroid cell culture (data not shown), are listed along with the reference samples in Table 1. Other UK sample submissions which were selected for investigation are listed in Table 3.

Results

Fluorogenic RT-PCR

(i) Testing of reference samples

The CT values and assay scores using the fluorogenic RT-PCR on ES and cell culture grown virus preparations of reference FMD and SVD viruses, on cell culture grown antigens of the other vesicular disease viruses of VS and vesivirus and from the negative controls are listed in Table 1. After

careful consideration of the TaqMan® amplification plots of the samples, threshold levels of either 0.200, 0.250 or 0.500 were consistently derived; the CT value for each PCR assay being the point on the x-axis (showing the number of cycles of replication) where the amplification plot intercepts the threshold line as illustrated in Figure 1. Reduction of the threshold lowers the CT value. Mean CT values for each ES or cell culture virus preparation were calculated from the CT values of the replicates of the cDNA samples tested in each PCR assay. ES or cell culture supernatants with a mean CT value of less than or equal to 38.50 were scored as positive, samples with the mean CT above 41.50 were considered negative and samples with mean CT values within 38.50 - 41.50 (i.e. CT value cut-off level of 40.00 +/- 1.50) were borderline and were re-tested (Table 1). Replicates of a sample with the same superscript adjacent to the CT value were from the same PCR amplification assay while CT values of a sample with different superscripts resulted from PCR amplifications in separate assays. The results of the fluorogenic RT-PCR from the reference samples are summarised in Table 2. The assay was specific for detection of FMD virus genome and detected all seven serotypes (Tables 1 and 2). All of the epithelial suspensions and cell culture grown virus preparations of the FMD viruses of serotype O, C, Asia 1 and SAT 3 scored positive and all of the serotype A strains were detected despite the negative result from one cell culture grown virus of this serotype (Table 1). The PCR was negative on one ES each of SAT 1 and SAT 2 viruses but all cell culture virus preparations of these serotypes were detected. Overall, the fluorogenic RT-PCR detected 57 of the 59 (97%) prepared ES and 70 of the 71 cell culture supernatant fluids (99%) of the reference FMD viruses. The other vesicular disease viruses of SVD, VS and vesivirus and the negative controls had CT values close to or at 50.00 and were thus all negative (14 out of 14 samples; 100%); demonstrating the specificity of the primers/probe for detection of FMD virus genome. Strong positive FMD samples had CT values below 20.00. The intra-assay

reproducibility was very good as illustrated by the closeness of the CT values from sample replicates and generally fulfilled the intra-assay acceptance criteria of the GeneAmp® 5700 Sequence Detection System manufacturer: namely, that the CT value between replicates tested in the same assay should differ by no more than 2.0-3.0 (Applied Biosystems, technical information). The inter-assay reproducibility was not as good but CT values between the replicates were sufficiently close to enable each virus to be scored in the same category (i.e. positive, negative or borderline for FMD virus genome). The amplification plots of all replicates were scanned to ensure the authenticity of the reported CT value as false positive values can arise from spurious, non-specific amplification of nucleotide sequence. This shows up as an erratic, non-sigmoidal amplification plot (Figure 2) rather than the true sigmoidal plot indicative of an authentic FMD-positive sample (Figure 1).

When the longer and shorter reverse transcription methods were used on the same samples of the serotypes SAT 1, SAT 2 and SAT 3, the longer protocol was clearly more efficient as the corresponding CT values were lower (Table 1).

(ii) Testing of the UK sample submissions which were positive for FMD virus both by ELISA and virus isolation on prepared ES

All of the FMD virus positive submissions from the UK 2001 outbreak which tested positive by ELISA and virus isolation on the prepared epithelial suspensions were positive by the fluorogenic RT-PCR (11 out of 11 samples [100%]; Table 1) although the TaqMan® amplification plots from these samples indicated that lower thresholds of 0.050 or 0.100 were more appropriate for the derivation of CT values. Strong positive samples produced CT values < 20.00 (Table 1).

Conventional RT-PCR

(i) Testing of reference samples

The results of the conventional RT-PCR procedure on the individual ES and cell culture virus preparations are also shown in Table 1 and the results summarised in Table 2. The conventional RT-PCR also detected all seven serotypes of FMD virus and all ES and cell culture virus preparations of the FMD serotypes O, A, Asia 1, SAT 1, SAT 2 and SAT 3 but only detected nine out of 12 (75%) of the serotype C FMD viruses. However, overall the conventional RT-PCR detected 53 of the 56 ES (95%) as positive.

(ii) Testing of a selected panel of samples submitted from the UK 2001 outbreak by the fluorogenic and conventional RT-PCR procedures, ELISA and virus isolation

Approximately 90% of positive epithelial samples received during the course of the UK FMD outbreak have been diagnosed by ELISA on the prepared ES. Completion of diagnosis on the remaining 10% required virus amplification in cell culture (this took from one to four days) before a diagnosis has been achieved. A panel of such samples has been selected for examination for RT-PCR and the results compared with those obtained by ELISA and virus isolation in cell culture. Table 3 shows the results of the fluorogenic and conventional RT-PCR procedures, ELISA and virus isolation on the selected samples. Several UK samples testing negative for FMD virus by each diagnostic procedure were included in Table 3 as controls. The sample UKG 15, 072/2001, also listed in Table 3, was unusual amongst the UK sample submissions as the ES prepared from this sample was positive on ELISA but failed to produce a recognisable CPE after two

passages in cell culture. The selected panel of FMD virus positive submissions from the UK 2001 outbreak were all positive by the fluorogenic RT-PCR (Table 3) and as with the UK sample submissions in Table 1, lower thresholds of 0.050 or 0.100 were more appropriate for the derivation of CT values. The UK sample UKG 15, 072/2001 was detected by both the fluorogenic and conventional RT-PCR procedures and all other UK samples were detected by the fluorogenic RT-PCR and produced a recognisable CPE in cell culture. The fluorogenic RT-PCR detected FMD virus in nine out of nine ES while the conventional RT-PCR detected eight out of nine positives. Only one of these nine ES samples were positive in ELISA while the other eight were positive by virus isolation. Thus, both RT-PCR procedures were more successful than ELISA for detection of FMD virus in ES. Intra- and inter-assay reproducibility of the fluorogenic RT-PCR on the selected panel of samples was again high and the RT-PCR negatives had CT values at or close to 50.00 (Table 3).

Table 1

Results of the fluorogenic and conventional RT-PCR procedures on epithelial suspensions (ES) and cell culture supernatant fluids (cc) of reference FMD virus samples and negative controls, ES prepared from UK sample submissions and cell culture supernatant fluids of other vesicular disease viruses (superscripts adjacent to each C_T value refer to the PCR assay number, mean C_T value for each assay on ES and/or cc of the samples is shown in brackets)

Sample	Country of origin	Sero-type[a]	CT values by fluorogenic RT-PCR		5'nuclease probe-based RT-PCR result[b]		Conventional RT-PCR[c]	
			ES[d]	cc[e]	ES	cc	ES	cc
TUR 1/80	Turkey	O		22.97[1] 23.22[1] (23.10[1]) 22.85[2] 23.03[2] (22.94[2]) 28.00[3] 28.42[3] (28.21[3]) 19.77[4] 20.20[4] (19.99[4])		+		+
TUR 3/87	Turkey	O	20.32[1] 20.49[1] (20.41[1])	26.37[1] 30.79[2] 31.07[2] (30.93[2])	+	+	+	+
KUW 3/88	Kuwait	O	18.33[1] 19.17[1] (18.75[1])	27.42[1] 27.28[1] (27.35[1])	+	+	+	+
SAU 8/88	Saudi Arabia	O	17.97[1] 18.20[1] (18.08[1])	27.57[1] 28.38[1] (27.98[1])	+	+	+	+
BRA 4/94	Brazil	O	22.98[1] 23.72[1] (23.35[1]) 22.70[2] 22.80[2] (22.75[2])	23.18[1] 23.41[1] (23.31[1])	+	+	+	+
GRE 21/94	Greece	O	22.75[1] 24.34[1] (23.54[1])	30.30[1] 23.86[2] 24.28[2] (24.07[2]) 24.21[3] 24.66[3] (24.44[3])	+	+	+	+
SAU 17/94	Saudi	O		19.98[1] 20.85[1] (20.42[1])		+		+[f]
SAU 72/94	Saudi	O	19.30[1] 19.88[1]	33.45[1] 33.59[1] (33.52[1])	+	+	+	+
TUR 3/94	Turkey	O	18.11[1] 18.32[1] (18.22[1]) 17.40[2] 17.49[2] (17.45[2])	29.02[1] 29.58[1] (29.30[1])	+	+	+	+
MAY 12/95	Malaysia	O	23.65[1] 23.83[1] (23.74[1])	21.23[1] 21.24[1] (21.24[1])	+	+	+	+

PHI 13/95	Philip-pines	O	24.23[1] 23.12[2] 23.50[2] (23.31[2]) 23.24[3] 23.27[3] (23.26[3])	24.40[1] 25.00[1] (24.70[1]) 19.12[2] 19.49[2] (19.31[2])	+	+	+	+
IRN 15/97	Iran	O	29.75[1] 30.00[1] (29.88[1])	27.55[1] 31.01[2] 31.96[2] (31.49[2])	+	+	+	+
KUW 4/97	Kuwait	O	17.86[1] 18.46[1] (18.16[1])	27.31[1] 30.89[1] (29.10[1])	+	+	+	+
LAO 1/2000	Laos	O	24.68[1] 25.15[1] (24.92[1])		+			
UKG 10/2001	United Kingdom	O	18.06[1] 18.76[2] 21.57[2] (20.17[2])		+		+	
UKG 11/2001	United Kingdom	O	17.89[1] 17.20[2] 18.53[2] (17.87[2])		+		+	
UKG 12/2001	United Kingdom	O	19.98[1] 20.03[2] 21.59[2] (20.81[2])		+		+	
UKG 31/2001	United Kingdom	O	16.82[1] 15.84[2] 17.42[2] (16.63[2])		+		+	
UKG 32/2001	United Kingdom	O	19.03[1] 17.90[2] 20.17[2] (19.03[2])		+		+	
UKG 33/2001	United Kingdom	O	18.33[1] 17.80[2] 19.31[2] (18.55[2])		+		+	
UKG 12, 547/2001	United Kingdom	O	19.31[1] 20.00[1] (19.66[1])		+		+	
UKG 15, 068/2001	United Kingdom	O	26.19[1] 34.80[1] (30.49[1])		+		+	
UKG 15, 081/2001	United Kingdom	O	19.17[1] 23.91[1] (21.54[1])		+		+	
UKG 15, 082/2001	United Kingdom	O	17.70[1] 18.69[1] (18.20[1])		+		+	
UKG 15, 083/2001	United Kingdom	O	21.46[1] 21.94[1] (21.70[1])		+		+	
TUR 10/88	Turkey	A	23.68[1] 23.83[1] (23.76[1])	28.73	+	+	+	+
SAU 17/92	Saudi Arabia	A	26.18[1] 26.81[1] (26.50[1])	31.42	+	+	+	+
SAU 19/92	Saudi Arabia	A	32.43[1] 32.89[1] (32.66[1])	26.04[1] 27.85[1] (26.95[1])	+	+	+	+
TUR 1/92	Turkey	A	23.12[1] 23.34[1] (23.23[1]) 26.50[2]		+		+	

BRA 10/93	Brazil	A	28.54[1] 29.06[1] (28.80[1])	35.97[1] 36.19[1] (36.08[1])	+	+	+[f]	+
BRA 11/93	Brazil	A	34.72[1] 36.94[1] (35.83[1])	27.29[1] 28.47[1] (27.88[1])	+	+	+	+
SAU29/93[g]	Saudi Arabia	A	33.63[1] 35.17[1] (34.40[1]) 35.34[2] 35.57[2] (35.46[2]) 41.34[3] 42.03[3] (41.69[3])		+			
MCD 6/96	Macedonia	A	24.20[1] 24.28[1] (24.24[1])	30.37[1] 31.12[1] (30.75[1])	+	+	+	+
IRN 1/97	Iran	A	27.10	24.27[1] 19.03[2] 19.95[2] (19.49[2])	+	+	+	+
MAY 10/97	Malaysia	A	24.88[1] 25.07[1] (24.98[1]) 24.34[2] 24.79[2] (24.57[2])	19.35[1] 19.50[1] (19.43[1])	+	+	+	+
ERI 3/98	Eritrea	A	32.56[1] 33.09[1] (32.83[1])	31.20[1] 32.21[1] (31.71[1]) 30.77[2] 31.70[2] (31.24[2])	+	+	+	+
GAM 46/98	Gambia	A	37.43[1] 38.07[1] (37.75[1]) 29.94[2] 31.03[2] (30.49[2])	39.49[1] 45.99[2] 47.13[2] (46.56[2])	+	-[h]	+	+
TUR 4/99	Turkey	A	33.75[1] 36.23[1] (34.99[1]) 19.80[2] 20.28[2] (20.04[2]) 20.41[3] 21.24[3] (20.83[3])	30.34[1] 31.04[1] (30.69[1]) 23.28[2] 24.36[2] (23.82[2])	+	+	+	+
Waldman	Germany, 1926	C	34.59	29.83[1] 31.22[1] (30.53[1])	+	+	+	+
Loupoigne	Belgium, 1953	C	23.49[1] 23.93[1] (23.71[1])	37.54[1] 35.73[2] 36.59[2] (36.16[2])	+	+	+	+
Resende	Brazil, 1955	C	31.02[1] 31.29[1] (31.16[1])	27.23[1] 27.37[1] (27.30[1])	+	+	+	+
BEL 1/69	Belgium	C	25.90[1] 26.91[1] (26.41[1])	26.7[1] 21.74[2] 22.25[2] (22.00[2])	+	+	-	-
LEB 9/69	Lebanon	C	24.14[1] 24.86[1] (24.50[1])	26.83[1] 29.71[2] 34.67[2] (32.19[2])	+	+	+	+
C_3 Indaial	Brazil	C		18.97[1] 19.21[1] (19.09[1])		+		+
ANG 3/73	Angola	C	34.99[1] 36.46[1] (35.72[1])	25.35[1] 26.22[1] (25.79[1])	+	+	-	+
SAU 1/84[i]	Saudi Arabia	C	34.75[1] 35.23[1] (34.99[1])	18.97[1] 19.13[1] (19.05[1])	+	+	+	+
SRL 1/84[i]	Sri Lanka	C	19.76[1] 19.80[1] (19.78[1])	19.69[1] 19.71[1] (19.70[1])	+	+	+	+
PHI 3/87	Philippines	C	26.02[1] 27.05[1] (26.54[1])	26.50[1] 21.10[2] 21.43[2] (21.27[2])	+	+	+	+

ITL 2/88	Italy	C	39.50[1] 40.30[1] (39.90[1]) 31.83[2] 31.85[2] (31.84[2])	38.72[1] 39.64[1] (39.18[1]) 28.44[2] 29.27[2] (28.86[2])	+[f]	+[f]	+	+
SRL 4/88	Sri Lanka	C	29.29[1] 29.30[1] (29.30[1])	30.46[1] 30.58[1] (30.52[1])	+	+	+	+
ITL 3/89	Italy	C		22.09[1] 22.14[1] (22.12[1])		+		+
PHI 2/90	Philip-pines	C	41.11[1] 42.78[1] (41.95[1]) 31.93[2] 32.24[2] (32.09[2])	28.50[1] 24.97[2] 25.33[2] (25.15[2])	+[f]	+	-	+
BAN 2/92	Bangla-desh	C		19.29[1] 20.06[1] (19.68[1]) 18.53[2] 18.75[2] (18.64[2]) 21.58[3] 21.73[3] (21.66[3]) 20.29[4] 20.62[4] (20.46[4])		+		+
SR 2/58	South Africa	SAT 1		22.82[1] 23.54[1] (23.18[1])		+		
SA 13/61	South Africa	SAT 1		29.20 [1,j] 29.77 [1,j] (29.49 [1, j])		+		
ISR 4/62	Israel	SAT 1		28.21[1] 28.30[1] (28.26[1])		+		+
BOT 1/68	Botswana	SAT 1		27.84[1] 28.82[1] (28.33[1])		+		+
NIG 20/75	Nigeria	SAT 1	45.80 [1,j] 49.09 [1,j] (47.45 [1,j])	37.02 [1,j] 37.82 [1,j] (37.42 [1, j]) 35.29[2] 35.64[2] (35.47[2]) 40.31[3,j] 41.47[3,j] (40.89[3,j]) 36.21[4] 36.42[4] (36.32[4]) 37.21[5,j] 40.34[5,j] (38.77 [5, j])	-	+		+
UGA 8/78	Uganda	SAT 1		22.64[1] 23.55[1] (23.10[1])		+		+
SWA 5/80	Swazi-land	SAT 1	25.11[1] 25.27[1] (25.19[1])	27.42[1] 27.85[1] (27.64[1]) 28.55[2] 28.85[2] (28.70[2])	+	+	+	+
KEN 9/91	Kenya	SAT 1	40.55[1,j] 40.58[1,j] (40.57[1,j]) 28.84[2] 29.40[2] (29.12[2])	26.05[1] 26.21[1] (26.13[1])	+[f]	+	+[f]	+
ZIM P18/91[k] (GN 31)	Zimbab-we	SAT 1		25.16[1] 27.06[1] (26.11[1])		+		+
ZIM P25/91[k] (UR 1)	Zimbab-we	SAT 1		25.74[1] 26.08[1] (25.91[1])		+		+
KEN 11/91	Kenya	SAT 1		25.47[1] 25.62[1] (25.55[1])		+		+
TAN 2/96	Tanzania	SAT 1		27.33[1] 27.47[1] (27.40[1])		+		+
MOZ 22/78	Mozam-bique	SAT 2	28.38[1] 28.84[1] (28.61[1])	24.39[1] 24.87[1] (24.63[1])	+	+	+	+
RHO 10/80	Rhodesia	SAT 2	34.86[1] 35.14[1] (35.00[1])	24.71[1] 28.50[1] (26.61[1]) 32.35[2] 32.99[2] (32.67[2])	+	+	+	+

ZIM 5/81	Zimbab-we	SAT 2		22.69[1] 24.31[1] (23.50[1])		+		+
ZIM 5/97	Zimbab-we	SAT 2	44.25[1,j] 44.26[1,j] (44.26[1,j])		-		+	
ZIM 6/97	Zimbab-we	SAT 2	40.72[1,j] 41.56[1,j] (41.14[1,j]) 33.63[2] 34.47[2] (34.05[2])		+[f]		+	
BUN 7/99	Burundi	SAT 2	19.43[1] 19.87[1] (19.65[1])		+		+	
RHO 2/74	Rhodesia	SAT 3	29.66[1,j] 29.73[1,j] (29.70[1,j])		+		+	
SAR 12/80	South Africa	SAT 3	34.76[1,j] 35.18[1,j] (34.97[1,j])	36.91[1,j] 37.83[1,j] (37.37[1,j])	+	+	+	+
ZIM 1/84	Zimbab-we	SAT 3		23.48[1] 25.55[1] (24.52[1])		+		+
ZIM 2/84	Zimbab-we	SAT 3	37.62[1] 38.75[1] (38.19[1])	36.84[1,j] 36.86[1,j] (36.85[1,j]) 28.63[2] 28.69[2] (28.66[2])	+	+	+	+
ZIM P30/90 [k] (Cher 32)	Zimbab-we	SAT 3		23.00[1] 23.29[1] (23.15[1])		+		+
TUR 15/73	Turkey	Asia 1	21.08[1] 22.02[1] (21.55[1])	25.33	+	+	+	+
BUR 12/77	Burma	Asia 1	31.09[1] 32.14[1] (31.62[1])	30.18[1] 30.53[1] (30.36[1]) 30.87[2] 31.46[2] (31.17[2])	+	+	+	+
SAU 2/80	Saudi Arabia	Asia 1		16.56[1] 16.84[1] (16.70[1])		+		+
KUW 2/81	Kuwait	Asia 1	19.11[1] 19.18[1] (19.15[1])	26.83[1] 27.33[1] (27.08[1])	+	+	+	+
IND 10/82	India	Asia 1	24.03[1] 24.29[1] (24.16[1])	24.87	+	+	+	+
NEP 58/88	Nepal	Asia 1	26.01[1] 26.58[1] (26.30[1])	33.12[1] 34.95[1] (34.04[1])	+	+	+	+
CAM 1/90	Cambo-dia	Asia 1		21.27[1] 21.81[1] (21.54[1]) 19.93[2] 20.28[2] (20.11[2])		+		+
CAM 3/93	Cambo-dia	Asia 1	25.55[1] 26.82[1] (26.19[1]) 18.72[2] 19.77[2] (19.25[2])	25.41[1] 28.52[1] (26.96[1]) 21.03[2] 21.62[2] (21.33[2])	+	+	+	+
SAU 39/94	Saudi Arabia	Asia 1	23.49[1] 23.71[1] (23.60[1])	27.11	+	+	+	+
CAM 4/97	Cambo-dia	Asia 1		21.81[1] 21.99[1] (21.90[1]) 20.63[2] 21.72[2] (21.18[2]) 25.01[3] 27.51[3] (26.26[3]) 25.99[4] 26.27[4] (26.13[4])		+		

NEP 40/97	Nepal	Asia 1	19.42[1] 19.62[1] (19.52[1])		+		+	
PAK 2/98	Pakistan	Asia 1	30.66	26.76[1] 35.56[2] 36.33[2] (35.95[2])	+	+	+	+
PAK 3/98	Pakistan	Asia 1	24.22[1] 24.83[1] (24.53[1])	24.24[1] 24.49[1] (24.37[1])	+	+	+	+
IRN 58/99	Iran	Asia 1	23.78[1] 24.62[1] (24.20[1])	26.13	+	+	+	+
HKN 3/89	Hong Kong	SVDV [l]		45.95[1] 50.00[1] (47.97[1])		-		-
HKN 4/91	Hong Kong	SVDV	50.00[1] 50.00[1] (50.00[1])	50.00[1] 50.00[1] (50.00[1])	-	-	-	
ITL 8/94[g]	Italy	SVDV	50.00[1] 50.00[1] (50.00[1])	50.00[1] 50.00[1] (50.00[1])	-	-		
Colombia 1964	USA	VSV-NJ [m]	NA	48.93[1] 50.00[1] (49.47[1]) 49.80[2] 50.00[2] (49.90[2])	NA	-	NA	-
SMSV- 9[n]	USA	SMSV-9	NA	48.70[1] 50.00[1] (49.35[1])	NA	-	NA	-
Negative controls[o]			48.97[1] 50.00[2] 50.00[2] 50.00[3] 50.00[3] 49.90[4] 50.00[4] 47.08[5] 50.00[5] 44.73[6] 45.46[6] 50.00[7-12] 50.00[7-12]	49.41[1] 50.00[1] (49.70[1]) 50.00[2-13] 50.00[2-13] (50.00[2-13])	-	-	-	-

KEY

[a] Serotype of FMD virus unless otherwise stated.

[b] A mean C_T value < 38.50 denotes a positive result, a mean value > 41.50 denotes a negative result (assay repeated on samples with mean C_T values from 38.50 to 41.50).

[c] Conventional RT-PCR with O-type specific O_1 Kauf3800/P2 primer set on UK samples, conventional RT-PCR with the 1F/1R primer set on reference (non-UK) samples.

[d] ES, epithelial suspension.

[e] cc, cell culture supernatant fluid.

[f] Positive result on repeat testing.

[g] Original cell culture submitted for diagnosis instead of vesicular epithelium.

[h] Negative result on repeat testing.

[i] Original fluid submitted for diagnosis instead of vesicular epithelium.

[j] Shorter reverse transcription protocol (15 min at 37°C) performed to produce the cDNA sample. Otherwise the longer reverse transcription protocol (45 min at 37°C) was used to produce the cDNA sample (Reid et al., 1999).

[k] Original probang from Zimbabwe.

[l] SVDV, swine vesicular disease virus.

[m] VSV-NJ, vesicular stomatitis virus, type New Jersey.

[n] SMSV-9, San Miguel sea lion virus, serotype 9.

[o] ES prepared from uninfected bovine epithelium or from supernatant fluid of uninfected primary calf thyroid cell culture or IB-RS-2 cell line.

NA, not available for testing.

+, positive result.

-, negative result.

Table 2

Summary of the fluorogenic RT-PCR results and their comparison with the conventional RT-PCR assay on reference FMD viruses of all seven serotypes, cell culture grown virus preparations of the other vesicular viruses and negative controls

RT-PCR procedure	Ratio of number of positive samples to total number of samples tested per FMD virus serotype								
	FMD virus serotype								
	O	A	C	SAT 1	SAT 2	SAT 3	Asia 1	Total[a]	Other virus[c]
(A) Epithelial suspensions									
Fluoro-genic	12/12	13/13	12/12	2/3	4/5	3/3	11/11	57/59	0/2
Convent-ional[d]	11/11	12/12	9/12	2/2	5/5	3/3	11/11	53/56	0/1
(B) Cell culture									
Fluoro-genic	13/13	10/11	15/15	12/12	3/3	4/4	13/13	70/71	0/5
Convent-ional[d]	13/13	11/11	14/15	10/10	3/3	4/4	12/12	67/68	0/3

KEY

[a] Ratio of the total number of positive results to total number of samples tested of epithelial suspensions of all seven FMD virus serotypes.

[b] Ratio of the total number of positive results to total number of samples tested of cell culture supernatant fluids of all seven FMD virus serotypes.

[c] Other vesicular viruses of swine vesicular disease, vesicular stomatitis virus (serotype New Jersey), vesivirus (San Miguel sea lion virus serotype 9).

[d] Conventional RT-PCR with the 1F/1R primer set.

Table 3

Comparison of the results obtained by the fluorogenic RT-PCR, conventional RT-PCR, ELISA and virus isolation on epithelial suspensions prepared from field samples submitted for FMD virus diagnosis from the UK 2001 outbreak [a] (superscripts adjacent to each C_T value refer to the PCR assay number, mean C_T value for each assay on ES of the sample is shown in brackets)

Sample	Fluorogenic RT-PCR		Conventional RT-PCR[b]	ELISA	Virus isolation[c]
	C_T values	*Assay score*			
UKG 13/2001	49.52[1] 50.00[2] 50.00[2] (50.00[2])	-	-	-	NVI
UKG 14/2001	50.00[1] 50.00[2] 50.00[2] (50.00[2])	-	-	-	NVI
UKG 40/2001	50.00[1] 50.00[2] 47.96[2] (48.98[2])	-	-	-	NVI
UKG 41/2001	50.00[1] 42.06[1] 50.00[2] (46.03[2])	-	-	-	NVI
UKG 42/2001	50.00[1] 50.00[2] 50.00[2] (50.00[2])	-	-	-	NVI
UKG 43/2001	50.00[1] 50.00[1] (50.00[1])	-	-	-	NVI
UKG 44/2001	50.00[1] 50.00[1] (50.00[1])	-	-	-	NVI
UKG 45/2001	50.00[1] 50.00[1] (50.00[1])	-	-	-	NVI
UKG 122/2001	33.01[1] 34.24[1] (33.63[1])	+	+	-	+[d]
UKG 9, 197/2001	23.58[1] 24.03[1] (23.81[1])	+	+	-	+[d]
UKG 9, 342/2001	24.22[1] 25.68[1] (24.95[1])	+	+	-	+[e]
UKG 9, 407/2001	27.97[1] 29.41[1] (28.69[1])	+	+	-	+[d]
UKG 9, 443/2001	29.37[1] 30.42[1] (29.90[1])	+	+	-	+[d]
UKG 9, 542/2001	26.55[3] 27.08[3] (26.82[3])	+	+	-	+[e]
UKG 9, 634/2001	27.79[1] 28.53[1] (28.16[1])	+	+	-	+[d]
UKG 9, 918/2001	32.78[1] 34.68[1] (33.73[1])	+	-	-[f]	+[d]
UKG 12, 620/2001	50.00[1] 50.00[1] (50.00[1])	-	-	-	NVI
UKG 12, 632/2001	44.39[3] 50.00[3] (47.19[1])	-	-	-	NVI
UKG 15, 034/2001	50.00[1] 50.00[1] (50.00[1])	-	-	-	NVI

UKG 15, 072/2001	31.95[1] 32.56[1] (32.26[1])	+	+	+	NVI
UKG 15, 087/2001	50.00[1] 50.00[1] (50.00[1])	-	-	-	NVI
UKG 15, 088/2001	50.00[1] 50.00[1] (50.00[1])	-	-	-	NVI
UKG 15, 089/2001	50.00[1] 50.00[1] (50.00[1])	-	-	-	NVI
UKG 15, 094/2001	50.00[1] 20.25[1]	Repeat	-	-	NVI

KEY

[a] All samples submitted for diagnosis during the UK 2001 outbreak were either FMD virus serotype O or no FMD virus was detected.
[b] Conventional RT-PCR with the O-type specific O_1 Kauf3800/P2 primer set.
[c] FMD virus serotype O status confirmed by ELISA on supernatant fluid of cell cultures showing a recognisable CPE.
[d] CPE recognised after 48 hours.
[e] CPE recognised after 24 hours.
[f] ELISA result inconclusive.
NVI, no virus isolated.
+, positive result.
-, negative result or no virus detected.

Table 4

Nucleotide sequence, virus specificity, genomic location and size of PCR amplification product of the O/A/C/Asia 1 (1F/1R) and O_1 Kaufbeuren- 3800-3820/P2 primer sets used in conventional RT-PCR

Primer	Nucleotide sequence (5' - 3')	FMD[a] virus - serotype specificity	Genomic location[a]	Expected size of PCR amplification product (bp)
1F	GCCTGGTCTTTCCAGGTCT (SEQ ID NO: 4)	All serotypes	5' UTR[b]	328[c]
1R	CCAGTCCCCTTCTCAGATC (SEQ ID NO: 5)	All serotypes	5' UTR	
O_1 Kaufbeuren-3800-3820 (virus sense)	AAGAGGGCCGAAACATACTGT (SEQ ID NO: 6)	O	nucleotides 3800 to 3820	150[d]
P2 (anti-virus sense)	GAAGGGCCCAGGGTTGGACTC (SEQ ID NO: 7)	O	2A/2B, nucleotides 6 to 34	

[a] FMD, foot-and-mouth disease.

[b] UTR, untranslated region.

[c] 1F/1R primer set.

[d] O_1 Kaufbeuren- 3800-3820/P2 primer set.

Discussion

Fluorogenic RT-PCR methodology is becoming increasingly important for virus diagnosis of human diseases such as influenza virus and CMV where rapid and quantitative results are required from the processing of large numbers of samples without significant risk of contamination. The methodology can also be used for the diagnosis of FMD virus in field samples, particularly when large batches of samples are submitted for virus diagnosis over a prolonged period as has been the case with the UK 2001 outbreak. Samples submitted to the WRL for FMD virus diagnosis during the 2001 FMD epidemic were tested with the TaqMan® primers/probe along with reference field samples of each of the seven serotypes of FMD virus in order to provide an evaluation of fluorogenic RT-PCR methodology.

Reference samples of all seven serotypes of FMD virus were tested by the fluorogenic and conventional RT-PCR procedures. The fluorogenic procedure was more sensitive than the conventional method for detection of FMD virus serotype C (12 out of 12 samples detected as compared to nine out of 12 samples) and had a higher sensitivity for detection of serotype O/A/C/Asia 1 viruses (close to 100%) than for the detection of SAT 1 and SAT 2 serotypes (detected six out of eight ES but 27 out of 27 cell culture supernatant fluids as positive) which has also been demonstrated with the conventional RT-PCR procedure (Reid et al., 2000). Both RT-PCR procedures detected FMD virus in ES of SAT serotypes which was not detected by ELISA (data not shown). Each PCR method was specific for the detection of the FMD viral genome. For the detection of FMD virus in the panel of samples submitted from the UK 2001 outbreak, the fluorogenic RT-PCR successfully detected FMD virus in all 20 of the prepared ES which were either positive by ELISA (12 out of 20 samples) or which had

shown a recognisable CPE in cell culture (FMD virus specificity confirmed by ELISA; 19 out of 20 samples). The fluorogenic procedure also detected FMD virus in more ES of positive samples than was obtained by the conventional RT-PCR (detected only 19 of the 20 samples) thereby demonstrating the higher level of sensitivity achievable with TaqMan® primers/probe for PCR amplification compared to the single primer set used in the conventional RT-PCR.

In addition to the benefits of high sensitivity and specificity, this study demonstrated that a higher throughput of diagnostic samples was achieved with the fluorogenic RT-PCR than can realistically be achieved with the conventional RT-PCR without increasing the risk of contamination of RNA or cDNA samples or PCR products. One sample, UKG 15, 094/ 2001, produced a false positive result from one replicate (Table 3) but this was quickly detected as the sample, in common with others, was tested in duplicate by the fluorogenic RT-PCR. The method is more rapid than the conventional RT-PCR and there is no requirement to analyse PCR products by relatively insensitive gel electrophoresis. Unlike conventional RT-PCR, the fluorogenic RT-PCR provides a quantitative and objective measure of target RNA/DNA product which facilitates the definition of positive, negative and borderline samples in the test. The minimum CT value of 18.50 is equivalent to a concentration of 10^7 TCID per ml of serotype O, Asia 1 and SAT 3 FMD virus which is equivalent to approximately 10^{10} - 10^{11} RNA molecules per ml. The CT value of 40.00 chosen as the positive to negative cut-off equals around 1-10 TCID per ml (approximately 1000 - 100, 000 molecules of RNA per ml or 1-100 RNA molecules per µl; Zhidong Zhang, unpublished results; see also Oleksiewicz et al., 2001 and Alexandersen et al., 2001). Importantly, the fluorogenic RT-PCR had high intra- and inter-assay reproducibility which fulfilled the acceptance criteria

laid down by the manufacturer of the 5700 thermocycler (Applied Biosystems) and simplified the scoring of assay results.

It is envisaged that the fluorogenic RT-PCR will be used in conjunction with automated RT-PCR (robotic arm) technology to enable even more samples and/or replicates of samples to be tested in a single assay in order to speed up the diagnostic process from sample receipt to the production of CT values following PCR amplification (see Example 2 below). Not only would this be more convenient when many samples are submitted for FMD virus diagnosis, such as epithelial tissue, blood/serum or probangs from the UK 2001 outbreak, but the combined results of RT-PCR and ELISA (on ES) could be obtained within narrower time-scales. Probangs, like original blood/serum samples, cannot be tested directly by ELISA but must first be inoculated onto cell culture to yield a recognisable CPE (the FMD virus specificity must then be confirmed by ELISA) which may possibly take up to three days. Clotted blood or serum samples submitted for FMD virus diagnosis have been tested by automated fluorogenic RT-PCR with promising results (Reid et al., 2001). Therefore the fluorogenic RT-PCR methodology may be used as a screening tool on blood/serum samples to complement the virus isolation results.

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Example 2: Automated fluorogenic RT-PCR assay

Materials and methods

Sample preparation

Suspensions of vesicular epithelium (ES) submitted from the UK 2001 FMD epidemic were prepared, tested by ELISA (and by automated RT-PCR retrospectively) and inoculated onto primary calf thyroid cell cultures at the time of sample receipt (Ferris and Dawson, 1988). A permanent line of IB-RS-2 cells was additionally used in attempts to isolate virus from porcine sample submissions and from those of unknown species origin. ES was similarly examined from contemporary field samples received from countries overseas during the course of the study (Senegal, Republic of Ireland, Malaysia, Iran, Philippines, Bhutan and Iraq). Suspensions were additionally examined from four batches of "bone marrow" (from illegally imported pork, from illegally buried sheep, from bone marrow of bovine tail and the blood vessel of a bovine tail) and "offal" samples, all of which were submitted to the WRL for FMD to investigate for the presence of FMD virus.

Following inoculation with ES or other suspensions, cell cultures with a recognisable CPE on first passage were harvested to collect supernatant fluids for testing for the presence of FMD virus by ELISA or RT-PCR. Supernatant fluids from first passage cell cultures not showing a recognisable CPE were subjected to a second (blind) passage on fresh cell cultures and examined for evidence of CPE for a further 2 days.

Blood samples from the UK epidemic were commonly submitted in batch sizes ranging from 1 to >20 and were submitted for virological investigation

either with separate vesicular epithelial samples or as the sole sample type from the particular premises under disease investigation. Probang samples were occasionally submitted for virological investigation from flocks containing seropositive animals towards the end of the outbreak. Milk samples from two suspect dairy herds were also examined. The blood, milk and probang samples and sera taken from animals experimentally infected with the type O virus from the UK 2001 outbreak were normally inoculated onto primary calf thyroid cell cultures in a similar fashion to that for ES and other suspensions but a single passage of a maximum 72 hour duration was employed. However, one batch of milk samples from a suspected FMD-infected dairy herd was inoculated onto primary lamb kidney cell cultures.

Automated extraction of total nucleic acid from samples for RT-PCR

Immediately after sample preparation, 0.2 ml of ES or other suspension was added to 1 ml of TRIzol® Reagent (Life Technologies, UK) and stored at -70°C until the nucleic acid extraction procedures were undertaken. Blood, probang and milk samples were added to an equal volume of lysis/binding buffer (Roche, UK), vortexed for at least 10 to 15 sec and kept at room temperature until processed. Cell culture supernatants collected during the UK 2001 epidemic were also added to an equal volume of lysis/binding buffer for the extraction process but 0.2 ml of cell culture supernatant fluids following inoculation with ES prepared from Iraq and Bhutan samples were added to 1 ml of TRIzol® Reagent. For nucleic acid extraction, 200 µl of the sample solution in buffer was first added to one well of a sample cartridge (Roche) and once all 32 wells were filled, the cartridge was then placed inside a MagNA Pure LC (Roche) programmed to extract total nucleic acid from each sample to a final elution volume of 100 µl. Reagents from a MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, 192 isolations) were used and total nucleic acid extraction performed according

to the instructions supplied for the MagNA Pure LC programme: 'Total NA Serum_Plasma_Blood' and using reagent volumes dispensed by the robotic arm as outlined by the kit instruction manual (version 2, May 2001). One full vial each of Proteinase K reagent (reconstituted with elution buffer as per kit instructions) and magnetic glass particle suspensions (MGPs) were used per 32 samples and vortexed thoroughly before their addition. The kit reagents were added to the appropriate large or medium-sized reagent tub (Roche) and placed inside the MagNA Pure LC with the MGPs inserted last to minimise clumping of the glass particles prior to the extraction procedure. Extracted nucleic acids were left in the sample cartridge at 4°C inside the MagNA Pure LC if it had also been programmed to carry out the pipetting steps for a reverse transcription procedure immediately subsequent to nucleic acid extraction. Otherwise the nucleic acids were removed from the MagNA Pure LC and stored at -70°C in the sample cartridge until reverse transcription was performed on a later occasion.

Automated reverse transcription procedures

(i) Reverse transcription of a panel of 32 samples into a 32-well PCR plate

The MagNA Pure LC was programmed to add 9 µl of RT mix to each well of a 32-well PCR plate followed by the addition of 6 µl of each nucleic acid. The RT mix consisted of 1 x TaqMan® RT buffer, 5.42 mM MgCl2, 0.49 mM (each) dNTP, 0.39 U/µl RNase inhibitor, 1.23 U/µl MultiScribe reverse transcriptase (all supplied by Applied Biosystems) and random hexanucleotide primers (2.5 µM, Applied Biosystems or 1.0 µM, Promega). The RT process was completed by placing the PCR plate in a PTC-100TM thermal cycler (MJ Research, Inc.) and successively incubating at 48°C for 45 min, 95°C for 5 min and at 20°C for at least 20 min.

(ii) Reverse transcription of three consecutive panels of 32 samples into a 96-well PCR plate

The described total nucleic acid extraction procedure was carried out consecutively on three sets of 32 samples with each set of extracted nucleic acids being stored at -70°C before the reverse transcription procedure (each plate of nucleic acids was therefore subjected to at least one freeze thaw cycle). After placing a 96-well PCR plate inside the MagNA Pure LC, a specific programme allowed 9 µl of the described RT mix to be pipetted into the first 32 wells (positions 1 to 32) of the PCR plate, to be followed by the addition of 6 µl of each nucleic acid from the cartridge containing the first set of 32 nucleic acids into wells of positions 1 to 32 of the PCR plate. A second (and subsequent third) specific programme directed the transfer of 9 µl of a second (and then third) batch of freshly-prepared RT mix into wells of positions 33 to 64 (and wells 65 to 96 for the subsequent third transfer) of the PCR plate followed by the addition of 6 µl of each nucleic acid from a second (and then third) cartridge of 32 extracted nucleic acids into the wells from positions 33 to 64 (and, in turn, wells 65 to 96) of the PCR plate.

After all 96 nucleic acid extracts had been added to the RT mix, the PCR plate was removed from the MagNA Pure LC and the RT process completed as previously described.

PCR amplification by fluorogenic assay

(i) Design of primers and probe

Redundant primers and a fluorogenic 5' nuclease probe were designed from the 5' untranslated region of the virus genome for the intended detection of all seven FMD virus serotypes as described (see Example 1).

(ii) Manual pipetting of reagents for PCR amplification of a single batch of 32 samples

Following the automated programmes for nucleic acid extraction and reverse transcription, PCR amplification of sample cDNAs was carried out after 22 µl of PCR reaction mix was pipetted manually into a well of a MicroAmp® optical reaction plate (Applied Biosystems) followed by the manual addition of 3 µl of cDNA from the 32-well PCR plate. The PCR reaction mix contained 0.9 pmol/µl each of the forward and reverse primer, 0.3 pmol/µl of probe and 1 x TaqMan® master mix and the amplification was carried out in a GeneAmp® 5700 Sequence Detection System (Applied Biosystems) using the programme: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 sec, 60°C for 60 sec, 50 cycles (see Example 1).

(iii) Automated pipetting of reagents for PCR amplification of a single batch of 32 samples

An automated process for the PCR amplification of 32 samples was carried out by programming the MagNA Pure LC to pipette 18 µl of PCR reaction mix into an optical reaction plate followed by the addition of 7 µl of cDNA from the 32-well PCR plate. The PCR mix contained the same

concentrations of the primers and probe as described for the manual pipetting procedure and the PCR amplification was carried out as described.

(iv) Automated pipetting of reagents for PCR amplification of 96 samples ('fast RT-PCR protocol')

A programme to automate the PCR amplification of 96 samples in a single plate was also devised. Following the automated reverse transcription of three consecutive batches of 32 samples as described, the PCR plate containing 96 cDNAs was re-inserted into the MagNA Pure LC beside a MicroAmp® optical 96-well reaction plate. PCR reaction mix was prepared with the concentrations of the primers and probe as described and placed inside the MagNA Pure LC as directed by the programme. This programme allowed 18 µl of PCR reaction mix to be added to each well of the optical reaction plate and then transferred 7 µl of cDNA from each well of the PCR plate to the PCR reaction mix contained in the corresponding well of the optical reaction plate. PCR amplification was carried out as described.

Reproducibility of automated RT-PCR

The intra-assay reproducibility of the 96-well 'fast RT-PCR protocol' was consistently determined by incorporating FMD-positive and FMD-negative control samples within each of the three sets of 32 samples so that the reproducibility of the control results could be assessed at three distinct locations on the 96-well plate. Intra-assay reproducibility was further evaluated by testing a single cell culture grown virus preparation (UKG 12,189/2001) in all 96 wells by the described procedure. The nucleic acid extraction procedure was carried out on 32 replicates and the sample cartridge was subjected to all three rounds of the reverse transcription programme after the extraction procedure.

Inter-assay reproducibility of the 96-well 'fast RT-PCR protocol' was examined when the extracted nucleic acids from a batch of 32 (test and control) samples were tested on two separate occasions. Other FMD-positive and negative samples of prepared ES were tested by the 96-well 'fast RT-PCR protocol' in more than one assay run.

Results

RT-PCR assay acceptance criteria

A CT value (detection threshold; the cycle at which target sequence is detected) of 40.0 was selected as the positive/negative cut-off based on the scatter diagrams of CT values obtained as a consequence of examining ES by the three automated RT-PCR procedures (Figure 3). All known negative samples had CT values higher than 40.0 (in fact close to or at 50.0) and most positive samples had a CT value below 40.0. A cut-off CT value of 40.0 established a sensitivity and specificity of the assay which was highly comparable to the established method of virus isolation in cell culture (Reid et al., 2001; see also Example 1). Samples with CT values from 39.0-41.5 were designated 'borderline' (and would require retesting by the RT-PCR to determine their positive/negative status). After amplification, the plots of all samples were scanned to ensure that each apparent positive sample had produced sigmoidal plots (see Example 1) so that a CT value could be assigned to each PCR reaction as described previously (Oleksiewicz et al., 2001; Alexandersen et al., 2001; see Example 1). Positive control and test samples should have a CT value less than 39.0 and negative controls and test samples should have a CT value at or close to 50.0. Strongly positive samples should have CT values well below 30.0 (Figure 3).

RT-PCR using automated programmes for total nucleic acid extraction and reverse transcription of 32 samples followed by either manual pipetting or automated programming for PCR amplification

(i) Manual pipetting procedure for PCR amplification

The results of the RT-PCR, ELISA and virus isolation on the ES, other suspensions, blood, milk and probang samples originating from the UK epidemic in 2001 and from abroad and on sera from animals infected experimentally with the UK 2001 epidemic FMD virus strain are summarised in Table 5 (section A). The fluorogenic RT-PCR detected FMD viral RNA in 9 ES samples which were negative by ELISA but positive in cell culture. Five ES samples had a CT value in the range 39.0 to 41.5 and so were interpreted as borderline. Four of these samples were negative by ELISA but produced specific CPE in cell culture. Another sample which was negative by both ELISA and virus isolation in cell culture produced CT values of 38.7 and 43.6 in one assay (sample tested in duplicate) and so was classified as a fifth borderline RT-PCR case. Two ES samples were positive by ELISA and virus isolation but negative by RT-PCR while conversely 2 ES were positive by RT-PCR but negative by ELISA and virus isolation. Two 'trace' results on ELISA were considered borderline.

Blood samples submitted from 17 separate premises (batch sizes ranging from 1 to 29) were tested by fluorogenic RT-PCR and virus isolation. Five blood samples from a single batch were positive by virus isolation, 4 of which were also positive by RT-PCR. The samples from the remaining 16 premises were negative both by RT-PCR and through cell culture passage. All 55 sera selected from experimentally infected animals were positive by both the automated RT-PCR procedure and virus isolation in cell culture.

Five probang samples had CT values in the range 41.8 to 46.5 by RT-PCR and were therefore considered negative. One probang sample was positive by virus isolation and negative by RT-PCR (CT value of 46.3) but one probang sample from a different premises which was negative by virus isolation was borderline by RT-PCR (CT value of 40.1).

No FMD virus was detected in the suspensions of the 25 "bone marrow" samples by either RT-PCR, ELISA or cell culture virus isolation procedures.

(ii) Automated pipetting procedure for PCR amplification

The results of the fully automated 32-well RT-PCR (using automated pipetting for PCR amplification), ELISA and virus isolation on ES, "offal", blood and probang samples are summarised in Table 5 (section B). All the samples examined had been submitted from the UK except for 13 vesicular epithelium samples originating from Iran (and which included samples positive for FMD virus serotypes O, A and Asia 1). Eight ES samples which were negative by ELISA but positive in cell culture were detected by the fluorogenic RT-PCR while another 2 ES samples (1 from the UK and 1 from Iran) were positive by RT-PCR but negative by ELISA and virus isolation. One ES was borderline by RT-PCR but negative by ELISA and virus isolation.

Twenty six blood samples from 5 premises were tested by RT-PCR in comparison with virus isolation in cell culture. The RT-PCR gave positive and borderline results respectively with 2 samples (from a single premises) which were both positive in cell culture. The RT-PCR also gave a borderline result on a sample from another premises which had not shown a CPE in cell culture but other blood samples from these premises (but not

tested by RT-PCR) were positive by virus isolation. One ELISA result was borderline.

Of 20 probangs which were negative in cell culture, 1 sample tested borderline by RT-PCR (CT value of 39.0) and 2 others had CT values of 42.6 and 44.2 respectively and were thus considered negative.

No FMD virus was detected in both "offal" samples tested by RT-PCR or virus isolation.

RT-PCR using automated pipetting of reagents for PCR amplification of 96 samples ('fast RT-PCR protocol')

The results of the 96-well 'fast RT-PCR protocol', ELISA and virus isolation on ES, other suspensions, blood and probang samples were generally in close agreement and are summarised in Table 6. ES of 2 UK samples were negative by RT-PCR but positive both by ELISA and virus isolation. Two UK samples positive by ELISA but from which no virus was isolated in cell culture were positive and borderline, respectively, by RT-PCR. ES of another UK sample, 2 Iran, 3 Bhutan and 6 Iraq samples were positive by RT-PCR but negative both by ELISA and virus isolation. A sample from Malaysia was negative both by RT-PCR and ELISA but showed a CPE after inoculation of the ES in cell culture. The 'fast RT-PCR protocol' clearly detected more of the positive sample submissions than ELISA (8 'trace' results by ELISA were considered borderline) and was positive on more ES than virus isolation.

Blood samples from 40 separate premises (batch sizes ranging from 1 to 15 samples) were tested by both RT-PCR and virus isolation. The 'fast RT-PCR protocol' detected FMD virus in 1 more blood sample than the

combined procedure of virus isolation and ELISA (Table 6). Blood samples positive by virus isolation were generally positive by the RT-PCR although the RT-PCR was negative on 2 samples from a single premises which were positive by virus isolation.

Only one of the probang samples which were examined by the described RT-PCR procedure was positive by virus isolation. This sample gave a borderline result by RT-PCR as did another sample submitted from a different premises and which had been negative by virus isolation.

Performance of the automated RT-PCR procedures on cell culture supernatant fluids

Table 7 shows the results obtained by the 32-well RT-PCR using automated programmes for total nucleic acid extraction and reverse transcription, followed by manual pipetting (section A) or by the 96-well 'fast RT-PCR protocol' (section B) for PCR amplification on supernatant fluids collected from primary calf thyroid cell cultures or IB-RS-2 cells inoculated with ES or other suspensions in comparison with virus isolation in cell culture.

FMD virus was detected in 8 (section A) and 3 (section B) supernatant fluids from first passage cell cultures not exhibiting observable CPE (and consequently not examined by ELISA). CPE was observed in cell cultures in all except one instance when these samples were blind (second) passaged. However, this sample had been previously found to be positive by ELISA on ES. Thirty six (section A) and 41 (section B) cell culture supernatant fluids which did not produce a CPE after either first or second passage were clearly negative by RT-PCR examination of first passage cell culture supernatants. Thirteen supernatant fluids which produced a CPE on first passage were positive by the 'fast RT-PCR protocol' (Table 7).

Reproducibility of automated RT-PCR

A standard deviation across the plate of 1.58 CT units (mean CT value of 22.06) was calculated when a cell culture grown virus preparation of sample UKG 12,189/2001 was tested in all 96 wells by the 'fast RT-PCR protocol'. There was no significant divergence in CT values across the plate and there were no clear 'hot-spots' (negative CT values) in any well (data not shown). The inter-assay reproducibility of the 96-well 'fast RT-PCR protocol' was generally satisfactory as the CT values from both the re-testing of the batch of 32 samples/control nucleic acids and the re-testing of other selected samples by the entire 96-well 'fast RT-PCR protocol' procedure were in close agreement (data not shown).

Table 5

Comparison of the 32-well fluorogenic RT-PCR procedures using automated nucleic acid extraction and RT programmes (with manual pipetting for PCR amplification [section A] or with automated PCR amplification [section B]) with ELISA and virus isolation on suspensions of vesicular epithelium (ES), other suspensions, blood, milk, probang and other samples originating from the UK 2001 FMD epidemic and abroad and on sera from animals experimentally infected with the UK 2001 type O FMD virus strain

	Test procedure	Sample source	ES			Blood			Milk		Probang		Other[c]		
			+ve	-ve[a]	+/-?[b]	+ve	-ve	+/-?	+ve	-ve	+ve	-ve	+/-?	+ve	-ve
A	ELISA	Field[d]	54	59	2										
	Virus isolate	Field	69	46		5	131		0	20	1	54		0	25
	RT-PCR	Field	63	47	5	4	132	0	0	20	0	54	1	0	25
	Virus isolate	Experi-mentally infected animals				55	0								
	RT-PCR	Experi-mentally infected animals				55	0	0							
B	ELISA	Field	24	19	1										
	Virus isolate	Field	32	12		2	24								
	RT-PCR	Field	34	9	1	1	23	2			0	20		0	2

KEY

[a] '-ve', no virus detected.
[b] '+/-?', borderline result.
[c] 'Other', Bone marrow suspensions or offal samples.
[d] 'Field', field submission.

Table 6

Comparison of the automated fluorogenic 'fast RT-PCR protocol' (for the assay of 96 samples in a single plate) with ELISA and virus isolation on suspensions of vesicular epithelium (ES), other suspensions, blood and probang samples

Test procedure	ES			Blood			Probang			Other[c]		
	+ve	-ve[a]	+/-?[b]	+ve	-ve	+/-?	+ve	-ve	+/-?	+ve	-ve	+/-?
ELISA	104	104	8							0	3	
Virus isolate	124	92	0	26	226		1	159		0	3	
RT-PCR	134	80	2	27	220	5	0	158	2	0	3	

KEY

[a] '-ve', no virus detected.
[b] '+/-?', borderline result.
[c] 'Other', Bone marrow suspensions

Table 7

Performance of the 32-well fluorogenic RT-PCR procedure using automated nucleic acid extraction and RT programmes (manual pipetting for PCR amplification [section A]) and the 96-well fluorogenic 'fast RT-PCR protocol' (section B) on supernatant fluids from cell cultures inoculated with suspensions of vesicular epithelium or other tissue originating from the UK 2001 FMD epidemic and abroad

Test procedure		**Number of samples identified as positive (FMD virus serotype O by ELISA or FMD virus by RT-PCR) or negative following passage in cell culture**			
		First passage		*Second passage*	
		Positive	*NVD*[a]	*Positive*	*NVD*
A	Virus isolation	0/8	0/36	8/8	0/36
	RT-PCR	8/8	0/36	NT[b]	NT
B	Virus isolation	13/16	0/41	2/3	0/41
	RT-PCR	16/16	0/41	NT	NT

KEY

[a] NVD, no virus detected.
[b] NT, not tested.

Discussion

Automated programmes in a MagNA Pure LC combined with PCR amplification in a GeneAmp® 5700 Sequence Detection System enabled fluorogenic RT-PCR to provide FMD diagnostic results in a shorter time scale than either of our conventional RT-PCR or non-automated fluorogenic RT-PCR methods. Larger panels of field samples comprising suspensions of vesicular epithelium or other tissue, cell culture supernatant fluids, blood, milk and probang samples could be tested simultaneously in single runs to provide quantitative results. During the first 4 months of the UK 2001 epidemic the overall daily sample submissions ranged from a minimum of 2 (on day 5) to a peak of 263. In a typical 8 hour working day, one operator could generate results from 32 test samples by the automated 32-well RT-PCR procedures but 2 persons in tandem could realistically achieve 64 test results from these procedures in an extended working day (of 10 to 12 hours). The number could be increased to 96 samples using the 96-well 'fast RT-PCR protocol' by one person within 2 working days or by 2 people within a period of approximately 12 hours.

Automated RT-PCR detected virus in more FMD-positive samples of ES than ELISA in all three automated RT-PCR protocols. ELISA achieved a diagnosis on ES of approximately 90% of the total number of positive epithelium samples submitted during the UK 2001 epidemic (N. P. Ferris, unpublished results); the remaining 10% testing positive after sample passage in cell culture. Of the ES tested by the automated RT-PCR protocols in this evaluation which had tested positive for FMD virus by the combination of ELISA and virus isolation, 79% tested positive by ELISA alone (151 out of 191 samples) as opposed to 96% (183 out of 191 samples) by RT-PCR. The sensitivity of the 32-well RT-PCR for detection of FMD virus was close to that achieved by virus isolation in cell culture when

manual pipetting was used in the PCR amplification stage but higher sensitivity was achieved when the PCR amplification stage was also automated. This may have been due to the higher volume of cDNA (7 µl) transferred to the PCR mix in the automated pipetting procedure compared to the 3 µl of cDNA added manually. The automated pipetting of reagents in the PCR amplification was also likely to be more consistent and less prone to error. However, the 'fast RT-PCR protocol' used for the simultaneous PCR amplification of 96 samples performed as well as either of the 32-well RT-PCR procedures on ES. This 96-well protocol detected FMD virus in more blood samples than virus isolation in cell culture and had a high intra- and inter-assay reproducibility.

The ES samples testing positive by automated RT-PCR but negative by the combination of ELISA and virus isolation were probably true FMD-positive samples; for example, samples UKG 14, 965/2001 and IRN 54/2001 each tested positive by both the fully automated 32-well and 'fast RT-PCR protocol' RT-PCR assays and were also borderline and positive respectively by the other 32-well automated RT-PCR procedure described. Blood samples submitted with UKG 14, 965/2001 from the same FMD-suspected farm were positive by virus isolation and so the positive RT-PCR result for this UK sample may have been correct. Two samples from Iran (including IRN 542001), 3 from Bhutan and 6 from Iraq were negative by ELISA/virus isolation but were similarly submitted in batches containing positive samples and so may have contained FMD virus genome. This apparent higher sensitivity of the RT-PCR on ES (and on blood) could have been due to a lack of live virus in the samples leading to a failure to isolate virus combined with the lower detection threshold of the ELISA. The samples could have been contaminated but the automated pipetting procedures and the closed-tube system of the fluorogenic assay (the tubes do not need to be opened after PCR amplification) should make this unlikely.

Automated RT-PCR was able to achieve an accurate diagnostic classification of samples at the first passage cell culture point (i.e. after 2 days incubation) both for samples which were positive on second cell culture passage but not on first (judged on the basis of recognition of a CPE) and others which were negative through two sequential cell culture passages (Table 7). The number of such samples examined was low but the results suggested that automated RT-PCR may obviate the need for the standard routine second cell culture passage and thus reduce the maximum time-span for issue of results from 4 to 2 days.

Automated RT-PCR protocols could possibly be adjusted to achieve a higher degree of sensitivity. Specific programmes could be devised to achieve a greater concentration of nucleic acid from the extraction procedure. The described extraction procedure produced nucleic acid of a high purity at an elution volume of 100 µl of nucleic acid from 200 µl of sample (in buffer). Extraction programmes yielding 50 µl of nucleic acid from the same initial volume of sample or from a higher starting sample volume, say 400-500 µl, could be evaluated to see whether an increase in test sensitivity resulted. The intra- and inter-assay reproducibility of automated RT-PCR was shown to be good. This can be used to increase the flexibility of the automated procedures as test and control samples can reliably be processed in replicates instead of in single wells to allow the protocols to run fewer samples.

The universal primer/probe set used in this evaluation successfully detected FMD virus serotype O in samples submitted for diagnosis from the UK 2001 epidemic and FMD virus types O, A and Asia 1 in contemporary sample submissions from overseas. The same primer/probe set detected FMD viruses of all 7 serotypes in our non-automated fluorogenic RT-PCR

method to provide a universal detection of FMD virus (see Example 1). This primer/probe set would be extremely useful for diagnostic purpose in a similar circumstance to that for the recent, extensive FMD outbreak in the UK which involved one virus strain.

The results presented suggest that a definitive result might be achieved by RT-PCR at the end of one cell culture passage. However, the sensitivity of the 96-well 'fast RT-PCR protocol' was at least equivalent to that of virus isolation in cell culture. One could therefore envisage that the combination of ELISA and virus isolation in cell culture together with RT-PCR would only be required for the confirmation of a first outbreak of FMD in a previously FMD-free country. If a prolonged outbreak is then a consequence of the first incursion then the RT-PCR might either be performed automatically on each sample or only on those samples shown initially to be negative by ELISA. Either approach would obviate the necessity for the passaging of samples in cell culture and allow the issue of laboratory test results to be considerably accelerated.

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Example 3: Evaluation of a real-time PCR machine for rapid and portable diagnosis of foot-and-mouth disease

In the present example, studies were performed to evaluate the utility of a portable, real-time PCR machine, the Cepheid SmartCyler (SC) (Cepheid, 1178 Bordeaux Drive, Sunnyvale, California 64089, USA) in diagnosing FMD using the fluorogenic RT-PCR assay of the present invention

Materials and Methods

Nasal swab and serum samples were taken from 6 sheep inoculated with Type O UKG 2001 FMD (sheep UJ0, UJ1, UJ3, UI96, UI97 and UI99) and 4 sheep (UJ2, UJ4, UJ5 and UI98) housed together with the inoculated animals. The experimental details and full results have been provided elsewhere (Alexandersen et al., 2001). Samples were taken daily from day 0 (prior to inoculation) to day 13, although only even-day samples to day 10 were included in this investigation. Nucleic acid was extracted from the samples using Magna Pure LC Technology (Roche) and the RNA converted to cDNA via reverse transcription (Applied Biosystems TaqMan RT reagents) according to the manufacturer's instructions. cDNA was analysed initially on the Applied Biosystems' GeneAmp 5700 PCR machine (AB 5700) using the protocol currently being utilised for FMDV diagnosis as previously described (Reid and others, 2001). Briefly, 1μL or 3μL of cDNA from serum and nasal swabs respectively was added to 24μL/22μL of a PCR mix containing 0.2pmol/μL of probe SA-multi-P-IR-292-269R, 0.9pmol/μL of both forward primer SA-IR-219-246F and reverse primer SA-IR-315-293R (probe and primers from AB) and 1 x TaqMan mastermix (AB) for a final reaction volume of 25μL. PCR was carried out on the AB 5700 machine under the program: 50°C for 120secs, 1 cycle; 95°C for 600secs, 1 cycle; 95°C for 15 secs, 60°C for 60secs, 50 cycles. Data were

analysed using GeneAmp software and Ct values (cycle threshold values, the cycle at which the fluorescence of the sample reaches a certain threshold and is deemed to be positive) were calculated using a threshold of 0.1.

Identical cDNA was also analysed on the SC PCR machine using PCR beads (Amersham Pharmacia), in the first instance, as the base of the mastermix. The protocols and reagent concentrations used for the initial analysis on the AB 5700 were optimised using epithelial cell samples (known to be positive for UK 2001 FMDV) to determine the conditions under which the SC performed optimally (data not shown). The optimised reaction mix contained the appropriate volume of cDNA (1 or 3μL) in a mix containing 17/15μL RNase-free water, 1 PCR bead (providing dNTPs at 0.2 mM, KCl at 50 mM, $MgCl_2$ at 1.5mM, Tris-HCL (pH 9.0) at 10 mM and 1-1.5 units Taq DNA polymerase), 2mM $MgCl_2$, 0.8pmol/μL of both forward and reverse primers and 0.2pmol/μL of probe (final volume 25μL). Limitations on sample volume required some nasal swab cDNA samples to be diluted first with 5μL water before aliquots could be taken, but, given the exponential nature of amplification, this would have had minimal effects on the final Ct values obtained (as also observed on dilution series of positive standards run in parallel, see later). The optimal program on the SC was: 95°C for 120secs, 1 cycle; 95°C for 15secs, 60°C for 30secs, 50 cycles. Optical data were analysed using the SC software and Ct values calculated using a fluorescence threshold of 30 units. Each run included a water-only negative control, which gave a Ct of 50 (no virus detected) in all assays.

Results

The results of the assays are shown in Tables 8 and 9 for the nasal swab and serum samples respectively. These results demonstrate that whilst the SC produced comparable results for samples with high target cDNA content,

the assay was less effective in detecting weakly positive samples. The lack of sensitivity of the SC-beads based assay for detection of FMDV was shown by the reduced number of positive results (Ct value of 45 or less) obtained. Whilst the AB 5700 detected 20 and 28 positive samples for the serum and nasal swab samples respectively, the SC-beads assay detected only 11 and 9 such positives. Experiments were therefore carried out to improve assay performance and to get a more direct evaluation of the equipment's performance by using alternative PCR reagents (Applied Biosystems reagents).

A mastermix was created from a TaqMan core reagents kit (AB) containing 5.5mM $MgCl_2$, 0.2mM of each dATP, dCTP and a dGTP and 0.4mM of dUTP, 0.025U/μL of Taq Gold polymerase, 0.02U/μL UNG, 1 x buffer A, 1μL DNA and probe and primer concentrations as per the previous SC protocol. The samples were amplified in the SC under the program: 50°C for 120secs, 1 cycle; 95°C for 600secs, 1 cycle; 95°C for 15 secs, 60°C for 30secs, 50 cycles. Serial dilutions of FMDV plasmid (pT7S3) DNA were used for this analysis. Previous experiments identified the lower limit of detection of the SC-beads assay to be a 10^{-7} dilution, thus dilutions 10^{-5} to 10^{-9} were analysed on the SC using both beads and core reagents together with the appropriate running protocols. The optical data from this experiment is shown in Figure 4. The quality of the PCR products produced was additionally determined by gel electrophoresis (Figure 5). Figures 4 and 5 both illustrate the superiority of the SC-core reagents PCR product in both quantity and specificity, and also the sensitivity of the assay, with a lower detection limit of 10^{-9} compared to 10^{-7} for the SC-beads assay.

Thus, the use of PCR beads was unsuitable for the detection of FMDV on the SC machine due to a greatly reduced sensitivity.

Having shown that the SC-beads assay was inferior, it was then necessary to re-analyse the experimental samples with the AB core reagents to more accurately assess the potential of the SC machine. The nasal swab samples were chosen for re-analysis as these samples contained a much lower specific cDNA content and were thus a more stringent challenge for the SC. Reagents and protocols for this SC-core reagents assay were as mentioned above. Table 10 and Figure 6 illustrates that the SC-core reagents assay produced results comparable in accuracy and sensitivity to the AB 5700 and was superior of the PCR beads-based assay. In Figure 6 the reaction of the samples are given as 50-Ct (a total of 50 cycles done minus the threshold value) in order to make visual examination easier, as strong positive samples are shown as a high value and weak samples shown as a low value or zero (i.e a Ct value of 50).

Table 8

Ct values obtained from analysis of nasal swab samples on SC using PCR beads and corresponding results from the AB 5700 machine for comparison. A Ct value of 50 indicates that no virus was detected.

	DAY 0		DAY 2		DAY 4		DAY 6		DAY 8		DAY 10	
Animal	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*
UJ0	50	50	41.7	33.37	50	37.75	50	41.29	50	50	*50	50
UJ1	*50	50	50	50	50	38.73	50	44.04	50	50	*50	48.09
UJ2	*50	50	50	48.42	25.69	27.99	*42.92	33.51	50	40.53	50	48.65
UJ3	50	50	*50	32.4	36.72	40.66	50	40.27	50	46.82	*50	50
UJ4	50	50	50	41.58	50	40.3	*50	38.07	50	46	50	49.53
UJ5	50	50	50	50	50	34.87	*50	40.33	50	50	50	50
UI96	50	50	27.61	44.44	50	39.36	50	44.9	50	45.77	50	48.51
UI97	50	50	*31.63	30.48	34.58	34.5	50	44.22	50	50	50	50
UI98	50	50	50	50	50	34.53	*35.44	32.13	50	50	50	50
UI99	50	50	*50	31.85	37.81	38.17	*50	38.11	50	44.41	50	50

* indicates where samples were required to be diluted before aliquots could be taken, thus cDNA concentration was slightly lower.

Table 9

Ct values obtained from analysis of serum samples on the SC using PCR beads and corresponding results from the AB 5700 machine for comparison.

	DAY 0		DAY 2		DAY 4		DAY 6		DAY 8		DAY 10	
Animal	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*
UJ0	50	50	29.18	31.6	50	43.88	50	50	50	50	50	50
UJ1	*50	50	32.18	31.01	50	22.47	50	50	50	50	50	42.73
UJ2	*50	50	50	50	23.74	24.62	31.34	33.03	50	50	50	50
UJ3	50	50	25.2	27.02	50	35.73	50	50	50	50	50	50
UJ4	50	50	50	42.68	24.64	25.89	50	45.11	50	50	50	50
UJ5	50	50	50	49.66	33.88	34.53	50	39.38	50	50	50	50
UI96	50	50	26.45	33.48	50	35.83	50	50	50	50	50	50
UI97	50	50	26.81	30.42	50	41.46	50	50	50	50	50	50
UI98	50	50	50	50	27.66	33.14	50	41.25	50	50	50	50
UI99	50	50	28.66	36.27	50	50	50	50	50	50	50	50

* indicates where samples were required to be diluted before aliquots could be taken, thus cDNA concentration was slightly lower.

Table 10

Ct values from nasal swab samples obtained from SC assay using core reagents and corresponding results for same cDNA on the AB 5700 machine. * denotes diluted samples.

	DAY 0		DAY 2		DAY 4		DAY 6		DAY 8		DAY 10	
Animal	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*	*SC*	*5700*
UJ0	50	50	*37.12	33.37	40.38	37.75	41.04	41.29	50	50	50	50
UJ1	50	50	50	50	38.41	38.73	45.29	44.04	48.41	50	50	48.09
UJ2	50	50	50	48.42	33.25	27.99	34.27	33.51	41.63	40.53	41.6	48.65
UJ3	50	50	35.13	32.4	*38.34	40.66	38.22	40.27	*46.83	46.82	*43.6	50
UJ4	50	50	41.87	41.58	41.07	40.3	38.11	38.07	*50	46	50	49.53
UJ5	50	50	50	50	40.21	34.87	39.94	40.33	*50	50	50	50
UI96	50	50	36.79	44.44	36.76	39.36	41.59	44.9	50	45.77	50	48.51
UI97	50	50	34.98	30.48	35.77	34.5	50	44.22	50	50	50	50
UI98	50	50	*50	50	*40.13	34.53	35.24	32.13	50	50	50	50
UI99	50	50	38.63	31.85	*42.1	38.17	40.59	38.11	50	44.41	50	50

* indicates where samples were required to be diluted before aliquots could be taken, thus cDNA concentration was slightly lower.

Discussion

It has been shown that when the appropriate assay conditions were used, the SC real-time PCR machine provided a rapid and accurate method for FMDV diagnosis and may be a promising tool for promoting fast, and portable, diagnosis of FMD. The present results indicate that its sensitivity and specificity are comparable to previously established real-time PCR assays.

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CLAIMS

1. An isolated polynucleotide having the sequence of SEQ ID NO:1 or a fragment thereof, which fragment is capable of binding specifically to the complementary sequence of SEQ ID NO: 1.

2. A polynucleotide as claimed in claim 1 comprising a labelling moiety.

3. A polynucleotide as claimed in claim 1 or 2 comprising two labelling moieties.

4. A polynucleotide as claimed in claim 3 wherein one moiety can quench the fluorescence emission of the other moiety.

5. A polynucleotide as claimed in claim 3 or 4 wherein the moieties are bound to the polynucleotide at positions that allow one moiety to quench the fluorescence emission of the other moiety.

6. An isolated polynucleotide having the sequence of SEQ ID NO:2.

7. An isolated polynucleotide having the sequence of SEQ ID NO:3.

8. A method for determining whether a sample contains FMDV comprising:

(a) contacting the sample with a probe comprising a polynucleotide as claimed in any one of claims 1 to 5 *in vitro* under conditions that allow the probe to bind specifically to a target polynucleotide; and

(b) determining whether the probe has bound to a target polynucleotide;

thereby to determine whether the sample comprises a target polynucleotide, the presence of a target polynucleotide in a sample being indicative of presence of FMDV.

9. A method as claimed in claim 8 wherein the step of determining whether the probe has bound to a target polynucleotide comprises amplifying a region of the target polynucleotide, which region comprises the binding site of the probe.

10. A method as claimed in claim 9 wherein amplification is primed by a polynucleotide as claimed in claim 6.

11. A method as claimed in claim 9 or 10 wherein amplification is primed by a polynucleotide as claimed in claim 7.

12. A method as claimed in any one of claims 8 to 11 wherein the step of determining whether the probe has bound to a target polynucleotide comprises determining the fluorescence emissions of the probe.

13. A method as claimed in any one of claims 8 to 12 wherein the sample has been taken from a mammal.

14. A method as claimed in claim 13 wherein the mammal is selected from a pig, or a wild or domesticated ruminant such as cattle, a buffalo, a sheep, a goat or a deer.

15. A method for determining whether an organism is infected with FMDV comprising determining whether a sample from the organism contains FMDV by a method as claimed in any one of claims 8 to 14.

16. A method according to any one of Claims 8 to 15 wherein the method is automated.

17. A method of vaccinating an organism against FMDV comprising determining whether a sample from an organism contains FMDV by a method as claimed in any one of claims 8 to 16, and, if FMDV is not detected in the sample, administering an FMDV vaccine to the organism.

18. A method of treating an organism infected with FMDV comprising determining whether a sample from an organism contains FMDV by a method as claimed in any one of claims 8 to 16, and, if FMDV is detected in the sample, administering a therapeutic agent to the organism, which agent is effective in combating the FMDV virus.

19. A method for combating the spread of FMDV between organisms comprising determining whether a sample from an organism contains FMDV by a method as claimed in any one of claims 8 to 16, and, if the organism is infected with FMDV sacrificing the organism.

20. A method for determining whether a test vaccine is capable of preventing FMDV infection comprising administering the test vaccine to an organism, inoculating the organism with FMDV and determining whether a sample from the organism contains FMDV by a method as claimed in any one of claims 8 to 16.

21. A method for determining whether a test agent is capable of combating FMDV comprising administering the test agent to an organism infected with FMDV and determining whether the test agent is capable of combating FMDV by a method as claimed in any one of claims 8 to 16.

22. Use of a polynucleotide as defined in any one of claims 1 to 7 for detecting FMDV.

23. A system for detecting FMDV comprising a polynucleotide as claimed in any one of claim 1 to 5, wherein the specific binding of the polynucleotide to a target polynucleotide of a sample is indicative of the presence of FMDV in the sample.

24. A kit of parts comprising a polynucleotide as claimed in any one of claims 1 to 5, and optionally a polynucleotide as claimed in claim 6 and/or a polynucleotide as claimed in claim 7.

FIGURE 1

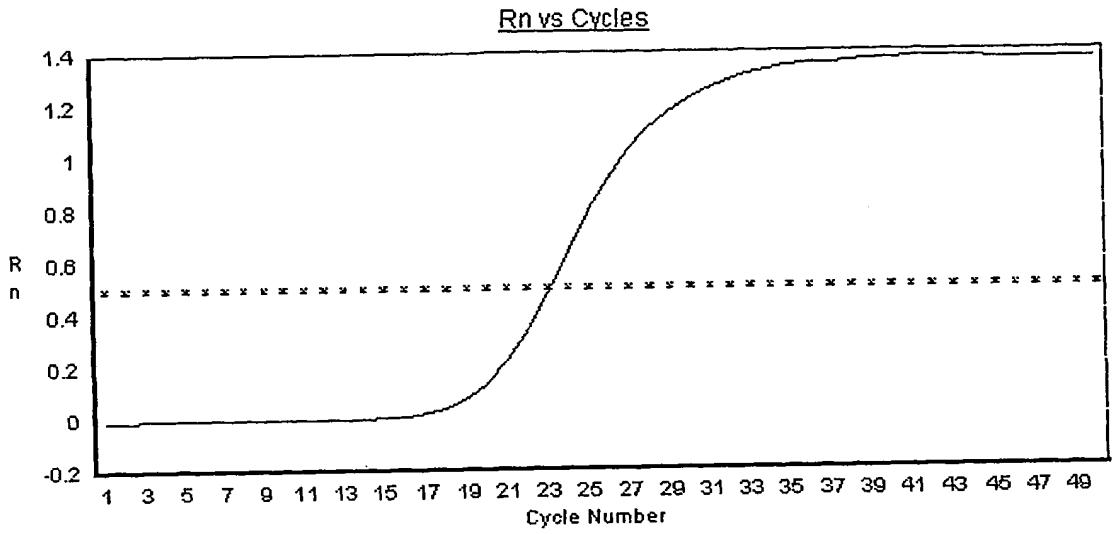
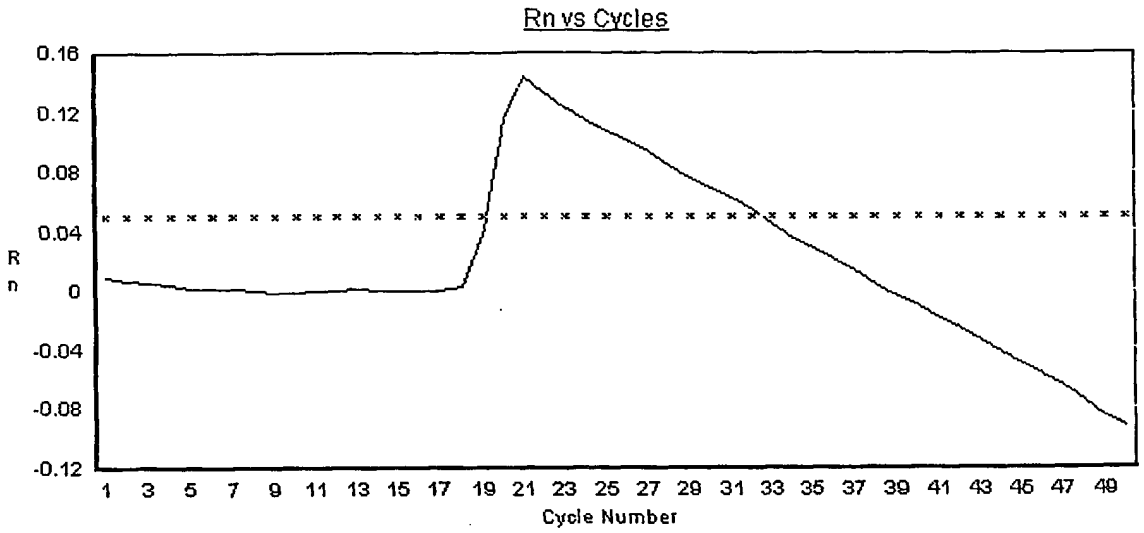

Rn vs Cycles
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
R
n
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
Cycle Number

FIGURE 2


Rn vs Cycles
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0.12
0.08
0.04
0
-0.04
-0.08
-0.12
Rn
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
Cycle Number

FIGURE 3(A)

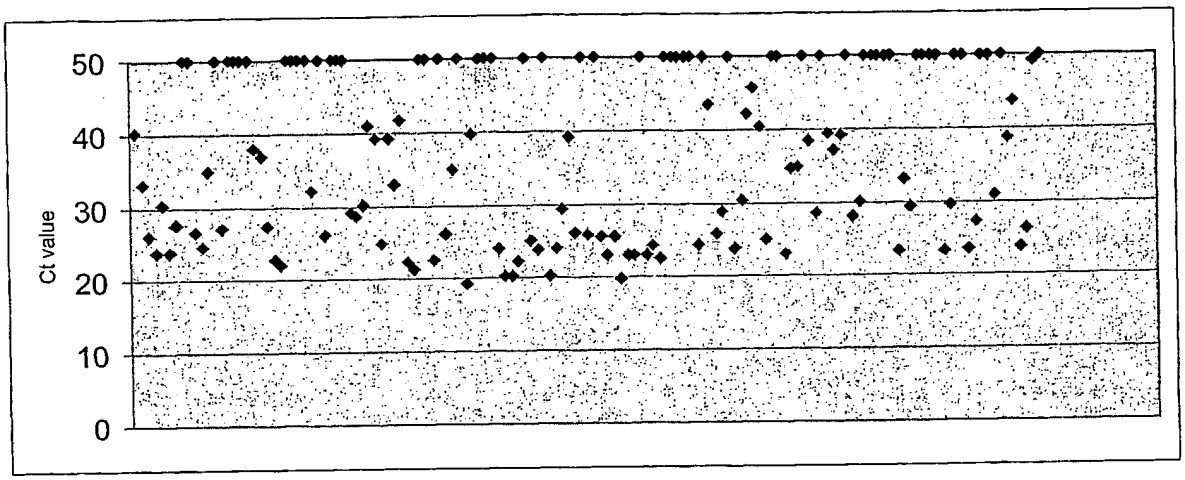
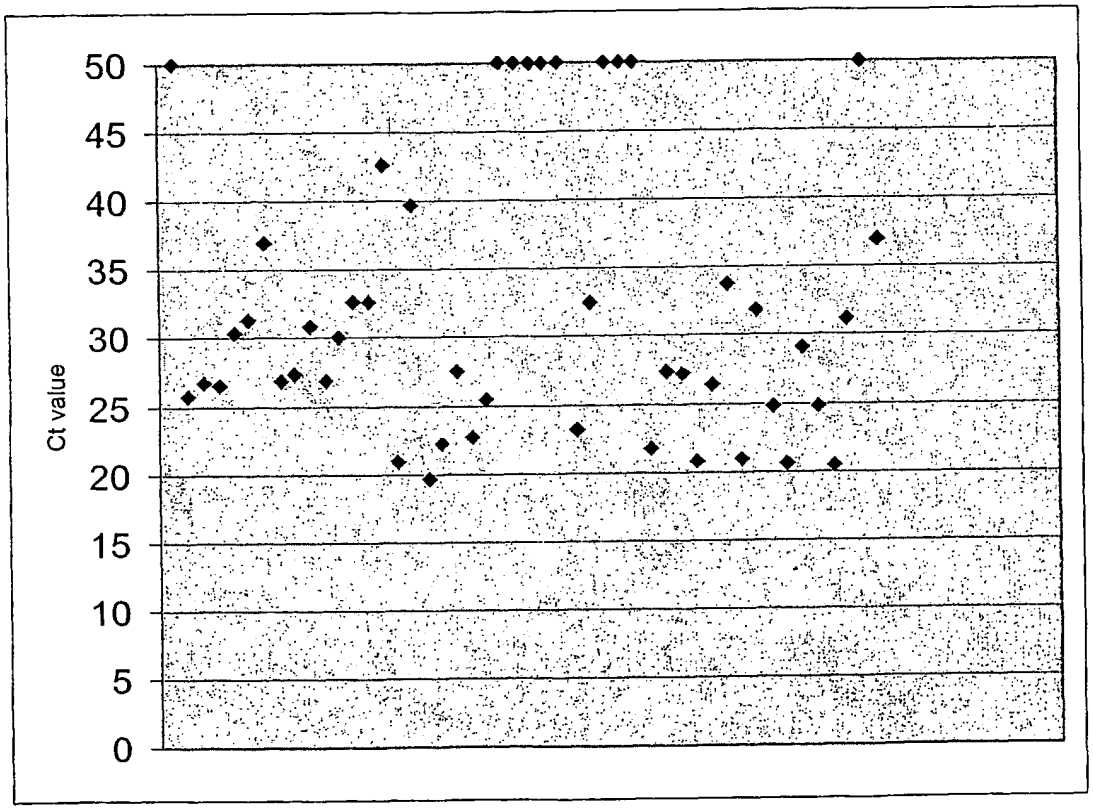


FIGURE 3(B)



5/9

FIGURE 3(C)

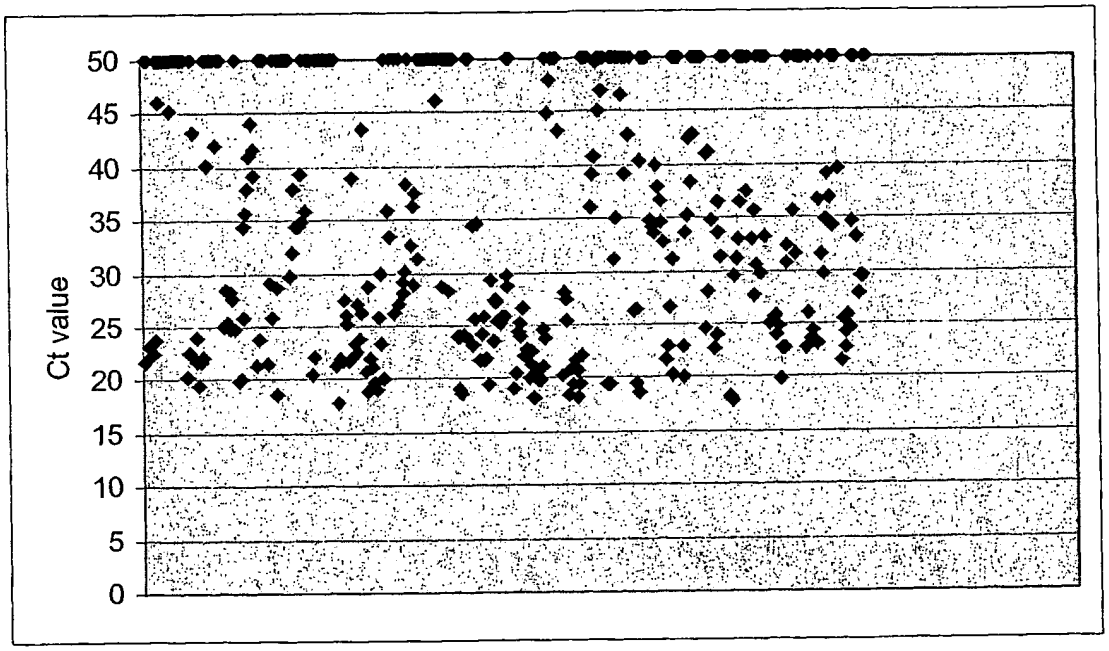


FIGURE 4

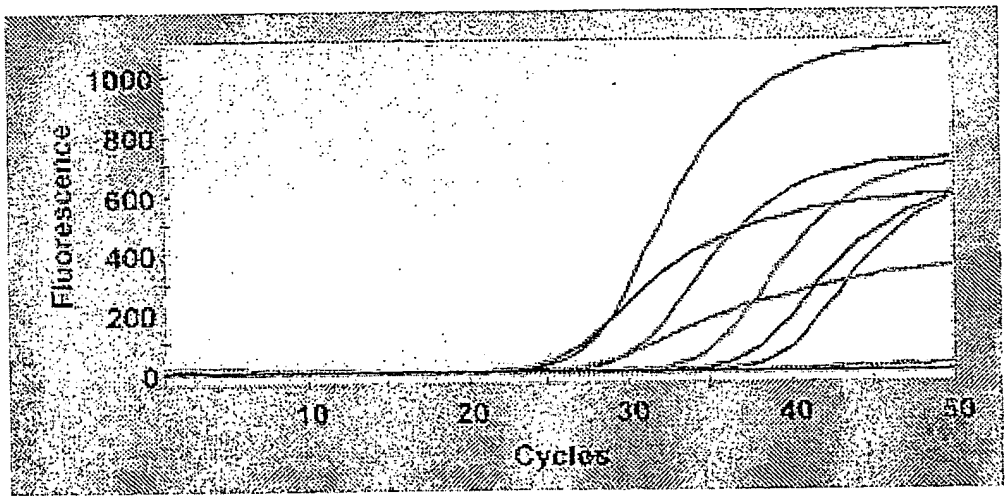


FIGURE 5

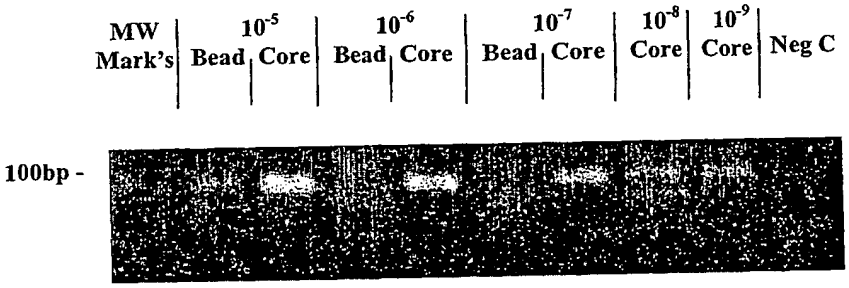

MW Mark's
10-5 Bead
Core
10-6 Bead
Core
10-7 Bead
Core
10-8 Core
10-9 Core
Neg C
100bp -

FIGURE 6 (A)

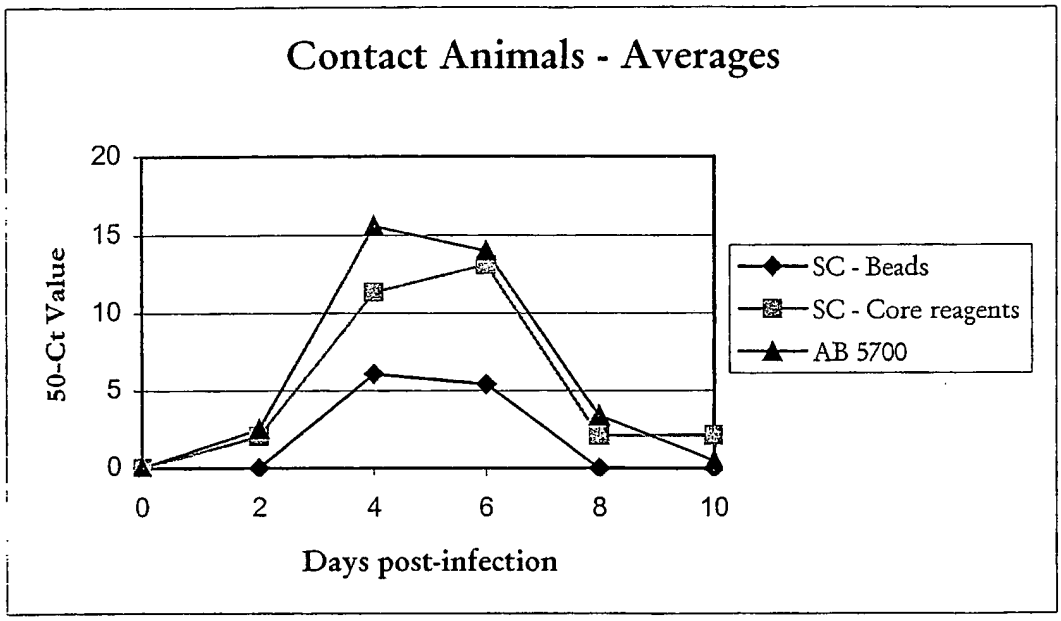
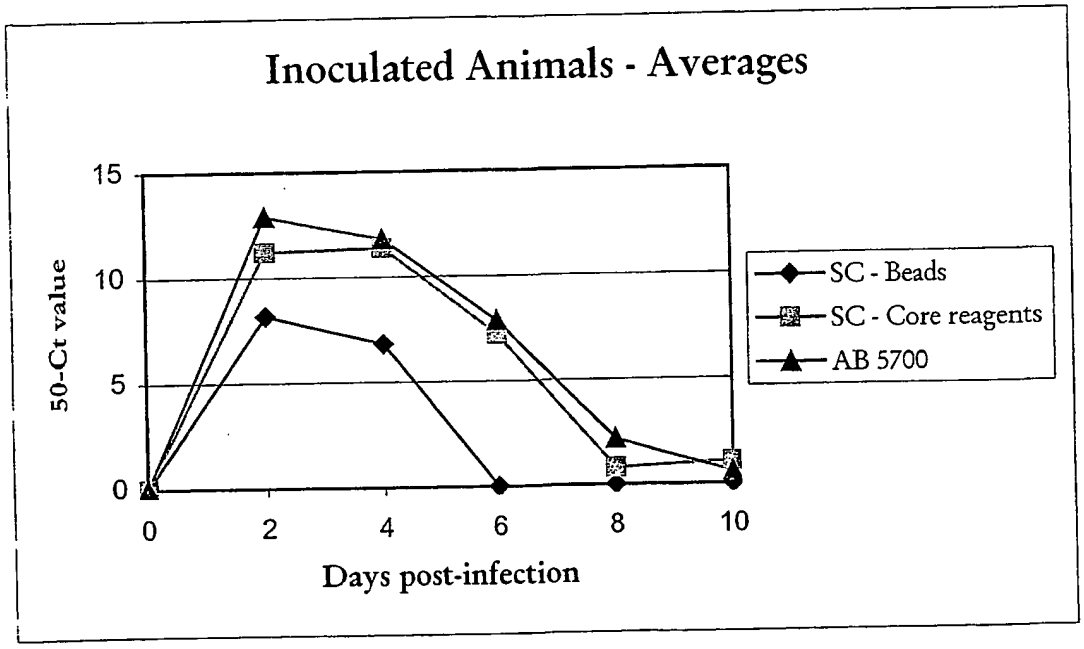

Contact Animals - Averages
50-Ct Value
20
15
10
5
0
0
2
4
6
8
10
Days post-infection
SC - Beads
SC - Core reagents
AB 5700

FIGURE 6 (B)


Inoculated Animals - Averages
50-Ct value
15
10
5
0
0
2
4
6
8
10
Days post-infection
SC - Beads
SC - Core reagents
AB 5700

SEQUENCE LISTING

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<120> ASSAY

<130> IAHA/P26494PC

<150> GB 0115777.5
<151> 28 June 2001

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **REID, Scott, Maclean** [GB/GB]; Flat 2, Times House, Nelson Street, Aldershot, Hampshire GU11 1HD (GB). **ALEXANDERSEN, Soren** [DK/GB]; 18 Bullswater Common Rd., Pirbright, Woking, Surrey GU24 0LZ (GB).

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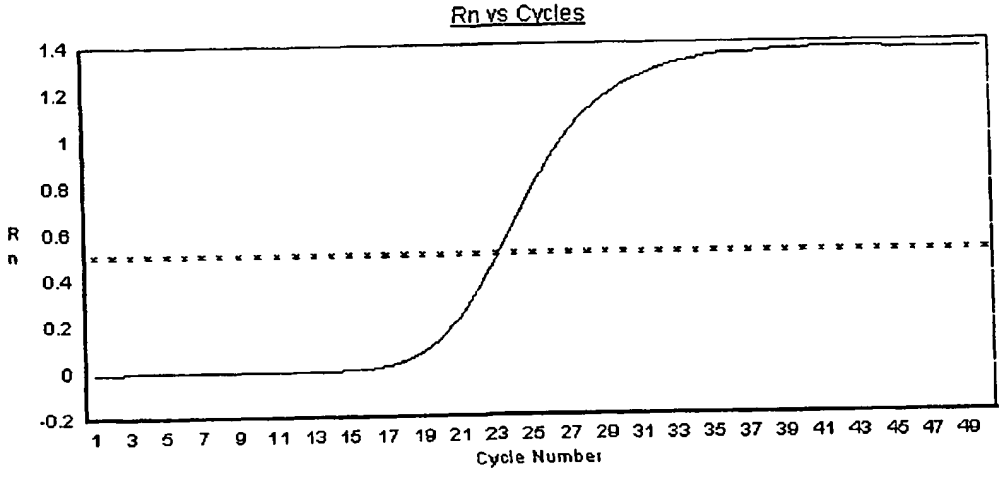
[Continued on next page]

(54) Title: METHOD FOR DETECTION OF FOOT-AND-MOUTH DISEASE VIRUS



(57) Abstract: The invention provides a polynucleotide having the sequence of SEQ ID NO:1 (CCTCGGGGTACCTGAAGGGCATCC) or a fragment thereof, which fragment is capable of binding specifically to the complementary sequence of SEQ ID NO: 1, a polynucleotide having the sequence of SEQ ID NO:2 (CAC{T/C}T{T/C}AAG{G/A}TGACA{T/C}TG{G/A}TACTG GTAC) and a polynucleotide having the sequence of SEQ ID NO:3 (CAGAT{C/T}CC{G/A}AGTG{T/A}C{I}C{I}TGTTA). The invention also provides a method for determining whether a sample contains FMDV comprising (a) contacting the sample with a probe comprising a polynucleotide having the sequence of SEQ ID NO:1 (CCTCGGGGTACCTGAAGGGCATCC) or a fragment thereof, which fragment is capable of binding specifically to the complementary sequence of SEQ ID NO: 1, *in vitro* under conditions that allow the probe to bind specifically to a target polynucleotide; and (b) determining whether the probe has bound to a target polynucleotide; thereby to determine whether the sample comprises a target polynucleotide, the presence of a target polynucleotide in a sample being indicative of presence of FMDV.

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(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/02943

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, MEDLINE, EPO-Internal, EMBL, EMBASE, BIOSIS, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 11016 A (GEN HOSPITAL CORP ;UNIV PITTSBURGH (US)) 2 March 2000 (2000-03-02) Sequence 22 (=GenSeq.AAA36764) has 88.9 identity with Seq. Id. No. 1 in 18 nt overlap '7-24:2-19! page 97 ---	1-5
X	DE 197 32 086 A (UNIV LEIPZIG) 28 January 1999 (1999-01-28) Primer #17 (=GenSeq. AAX02728) has 100% identity in 14 nt overlap with Seq.Id.No.1 '18-5:1-14! column 7 --- -/--	1-5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 June 2003	30/06/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Petri, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	REID S M ET AL: "Diagnosis of foot-and-mouth disease by real-time fluorogenic PCR assay." THE VETERINARY RECORD. ENGLAND 17 NOV 2001, vol. 149, no. 20, 17 November 2001 (2001-11-17), pages 621-623, XP009012159 ISSN: 0042-4900 page 621, column 2 the whole document	1-24
A	ALEXANDERSEN S ET AL: "Development of reverse transcription-PCR (oligonucleotide probing) enzyme-linked immunosorbent assays for diagnosis and preliminary typing of foot-and-mouth disease: a new system using simple and aqueous-phase hybridization." JOURNAL OF CLINICAL MICROBIOLOGY. UNITED STATES DEC 2000, vol. 38, no. 12, December 2000 (2000-12), pages 4604-4613, XP002244065 ISSN: 0095-1137 table 1	1-24
A	REID S M ET AL: "Primary diagnosis of foot-and-mouth disease by reverse transcription polymerase chain reaction." JOURNAL OF VIROLOGICAL METHODS. NETHERLANDS SEP 2000, vol. 89, no. 1-2, September 2000 (2000-09), pages 167-176, XP002244066 ISSN: 0166-0934 table 1	1-24
A	DATABASE EMBL 'Online! 7 November 1985 (1985-11-07) retrieved from EBI Database accession no. X00871 XP002244069	
P,A	REID S M ET AL: "Diagnosis of foot-and-mouth disease by RT-PCR: use of phylogenetic data to evaluate primers for the typing of viral RNA in clinical samples." ARCHIVES OF VIROLOGY. AUSTRIA DEC 2001, vol. 146, no. 12, December 2001 (2001-12), pages 2421-2434, XP002244067 ISSN: 0304-8608	

-/—

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/02943

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	REID SCOTT M ET AL: "Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay." JOURNAL OF VIROLOGICAL METHODS. NETHERLANDS AUG 2002, vol. 105, no. 1, August 2002 (2002-08), pages 67-80, XP002244068 ISSN: 0166-0934	

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/02943

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 17-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 02/02943

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0011016	A	02-03-2000	WO 0011016 A1		02-03-2000
			US 2003110526 A1		12-06-2003
DE 19732086	A	28-01-1999	DE 19732086 A1		28-01-1999

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